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MEASUREMENTS OF ACTIVE BIOMASS CONCENTRATIONS IN BIOLOGICAL WASTE TREATMENT PROCESSES

F. G. Pohland, et al

Georgia Institute of Technology
Atlanta, Georgia

September 1975

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MEASUREMENTS OF ACTIVE BIOMASS CONCENTRATIONS IN BIOLOGICAL WASTE TREATMENT PROCESSES

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FOREWORD

Man and his environment must be protected from the adverse effects of pesticides, radiation, noise, and other forms of pollution, and the unwise management of solid waste. Efforts to protect the environment require a focus that recognizes the interplay between the components of our physical environment--air, water, and land. The Municipal Environmental Research Laboratory contributes to this multidisciplinary focus through programs engaged in

- studies on the effects of environmental contaminants on the biosphere, and
- a search for ways to prevent contamination and to recycle valuable resources

As part of these activities, the study described herein presents an evaluation of the use of the determination of dehydrogenase enzyme activity for the accurate measurement of active biomass in both batch and continuous cultures grown on a variety of substrates in the biological wastewater treatment processes and may be a useful tool for the determination and close control of the biomass in the processes.

A. W. Breidenbach, Ph.D
Director
Municipal Environmental
Research Laboratory

ABSTRACT

This research was initiated to determine the applicability and limitations of the dehydrogenase test for the measurement of active biomass in biological wastewater treatment processes. Pure culture with E. coli and/or heterogeneous culture batch studies were conducted on a variety of substrates including glucose, galactose, sucrose, alanine, acetic acid, and selected industrial wastewaters. Also conducted were continuous aerobic or anaerobic culture studies with and without solids recycle. Dehydrogenase activity was monitored along with other parameters including plate count, Coulter Counter enumeration, adenosine triphosphate (ATP), and suspended solids to provide comparative and complementary information on the biomass concentration.

Dehydrogenase activity was a very sensitive and accurate measure of active biomass throughout the growth phases especially during endogenous growth but showed limitations with the nutrient deficient cultures. The correlation between dehydrogenase activity and suspend solids was constant at varying retention times, or at all growth rates with or without solids recycle. Consequently, a standard curve could be developed for given wastewaters by operating the measurement of active biomass and thereby effectively controlling the biological process.

The measurement of ATP was also a reliable new technique for measurement of active biomass except more study on the extraction method is required as well as investigations on the change of the correlation with suspended solids with the change of growth rate.

The technique for dehydrogenase activity measurement is simple, less costly and gives more reliable and interpretable results.

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This project was sponsored by the Environmental Protection Agency, with Dr. Ronald F. Lewis serving as Project Officer.

SECTION I

CONCLUSIONS

The use of dehydrogenase activity for the measurement of active biomass in the biological wastewater treatment process has proven very sensitive and effective in both batch and continuous cultures grown on a variety of substrates.

Definite relationships between plate count, Coulter Counter enumeration, solids and dehydrogenase activity were established during the log growth phase of batch cultures with E. coli.

Dehydrogenase activity is a more sensitive measure for active biomass under substrate limited endogenous growth.

ATP and dehydrogenase activity have shown a very similar pattern of measurements throughout the log and stationary phases of heterogeneous batch cultures except for lag observed during the anaerobic culture studies.

The correlation between dehydrogenase activity and suspended solids in continuous culture remained constant at all growth rates.

Except for nutrient deficient conditions where nitrogen and phosphorus are limiting, dehydrogenase activity was an acceptable measure of active biomass.

The correlation between dehydrogenase activity and suspended solids was constant but lower in continuous culture with solids recycle than without recycle. The existance of partial endogenous growth in the cultures did not affect this relationship.

When measured with wastes of different organic characteristics, the dehydrogenase activity per unit biomass changed in both batch and continuous culture.

The technique for dehydrogenase measurement is less costly and tedious than other biomass parameters such as ATP and yields more reliable and interpretable results.

SECTION II

RECOMMENDATIONS

Based on batch and continuous studies with pure and heterogeneous cultures under aerobic and anaerobic environments, it is recommended that pilot plant or field investigations at selected wastewater treatment plants be conducted to establish the validity and applicability of the correlations determined during these studies and to develop a standard techniques for the use of dehydrogenase activity measurements in the design and control of biological treatment processes.

SECTION III

REVIEW OF THE LITERATURE

Introduction:

The adequacies of available parameters for design and operation of water pollution control systems have become particurly apparent during recent years as requirements for increased treatment efficiency have intensified. Use of inappropriate analytical measurements has often resulted in poor performance which must be elminiated in order to meet current and projected demands for quality control. Biological monitoring procedures also suffer from such deficiencies and of those methods available (Figure 1), the determination of active biomass has become one of the most controversial.

One approach to the measurement of active biomass has been the adaptation of enzyme activity analysis to heterogeneous biological populations. In order to be successful, certain criteria must be satisified including:

1. a definite relationship between the gross enzyme concentration measured and the active (viable) biological population present in the system;

2. a similar enzyme concentration level for all bacterial species found in the system;

3. a resistance to variations in gross enzyme concentration with changes in environment or exposure to stress;

4. a methodology providing quantitative information not influenced by variations in the dissolved and suspended chemical constituents or physical characteristics of the system; and

5. a sufficient quantity of measurable indicator of enzyme activity to permit reasonable accuracy and precision.

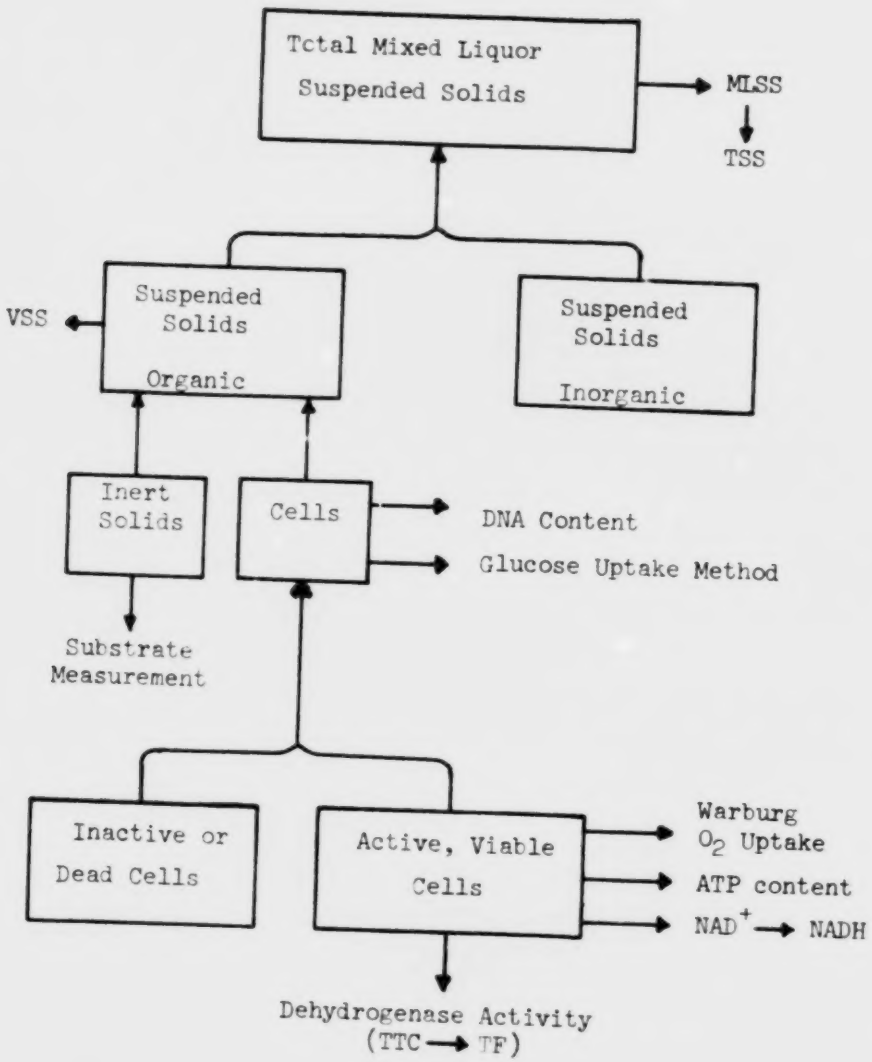


Figure 1. Biological Monitoring Tests (1)

Three parameters which have received consideration for adaption to biological treatment processes include the deoxyribonucleic acid (DNA) content of bacteria, the dehydrogenase activity of bacterial cultures and bacterial adenosine triphosphate (ATP) content. These methods are based upon several fundamental premises, viz., DNA is a nuclear constituent indigenous to all living cells; the dehydrogenases are vital to all biologically mediated reactions; and, bacterial ATP is associated only with living cells as a source of energy for biosynthesis and metabolism. Since the thrust of the research reported herein has been to further develop an adequate biochemical parameter to indicate active biomass content in biological waste treatment systems, a critical review of the available literature was considered in order. Similar reviews have been prepared by Patterson, et al.[2] and Weddle and Jenkins[3] in separate investigations. The former authors considered the measurement of cellular ATP superior to dehydrogenase activity determinations because of interferences of test reagents (TTC) with normal cell metabolism and difficulties in interpretation of results with the latter technique as compared with rapidity, simplicity and sensitivity of the ATP analysis. Conversely, the latter authors considered both parameters valid with ATP content remaining constant over a wide range of growth conditions. Similarly, the dehydrogenase enzyme activity as well as the oxygen uptake rate remained constant over a net growth rate range of 0.03-6.0 day^{-1} for the systems investigated.

DNA Methodology and Application:

General Perspective - Numerous papers have been published over the past twenty years on the quantitative determination of deoxyribonucleic acid (DNA) in various biological systems. Almost all cellular DNA ($\geq$ 95%) is found in the nucleus and distributed throughout the nucleoplasm as chromatin while the cell is in the resting state (i.e., engaged in maintenance and growth between cell division). In anticipation of division and during its course, the chromatin becomes highly organized into distinct linear structures called chromosomes. The number of chromosomes per somatic cell is constant and this constant complement is passed

on to a daughter cell as a result of mitotic division[4]. The microbial concentration of DNA has been shown to be invariant with physiological state and fairly constant among various bacterial species with an average of 2×10^3 µg/mg of cell material.[5,6]

DNA is a macromolecule which consists of a sequence of nucleotides linked together by phosphoric acid in a diester linkage; the nucleotides in DNA molecule consist of a 2-deoxy-D-ribose (carbohydrate) moiety with one of four bases (i.e., purines and pyrimidines) attached to the hydroxyl group on C-1' carbon atom. These four bases are thymine, cytosine, adenine, and guanine.[4,7]

Development of Analytical Methods - DNA determinations were originally made indirectly by gently heating an assay sample in dilute alkali so that the ribonucleic acid (RNA) would be degraded to acid soluble nucleotides allowing the DNA fraction to remain unhydrolyzed and to precipitate in dilute acid. By determining the carbohydrate and phosphate content of the acid-insoluble fraction, the initial DNA content could be calculated[7]. The ultraviolet absorption of the purine or pyrimidine moieties in the region of 270-357 mµ although specific requires the selection of suitable DNA standards since the proportion of purines to pyrimidines varies according to the source[8]. Webb and Levy[6] developed a colorimetric test for the direct measurement of the deoxyribose moiety of DNA with p-nitro-phenylhydrazine to yield a blue-colored product. DNA hydroloyzed in TCA (trichloroacetic acid) reacted quantitatively with p-nitrophenylhydrazine. When the product was separated from interfering substances, it was determinable colorimetrically in alkaline solution. The blue color developed in accordance with Beer's law over a range of 10 to 300 µg of DNA producing five times the color intensities and was more specific than the early diphenylamine reagent used in tissue and microorganism studies. It was postulated that the deoxypentose resulting from the hydroloysis forms a hydrazone when heated with the phenylhydrazine derivative in the presence of TCA. However, while the chromogenic compound was more intensely colored, the color started to fade almost immediately so that optical densities of all samples had to be recorded at approximately the same time. DNA

values on biological materials determined by reaction with p-nitrodiphenylhydrazine have been found to be generally lower than those values noted by reaction with diphenylamine reagent. These lower results may be explained by assuming that p-nitrodiphenylamine does not give color with protein or protein breakdown products resulting from TCA hydrolysis. It was observed, however, that in the presence of proteins and protein degradation products, the diphenylamine reagent did give high DNA values. Other carbohydrates present which might be capable of reacting with p-nitrodiphenylhydrazine either do not form colored products in alkaline medium or are extracted along with excess reagents by butyl acetate when in TCA solution thereby eliminating the need for any prior tissue or microorganism preparation. The DNA extracted from pure culture organisms yielded an average DNA content of bacterial cells of 3.8 percent on a dry weight basis.[6]

Agardy and co-workers[9-11] studied a method of determining the DNA content of anaerobic fermentation but detected a green color developed in the reaction with diphenylamine which tended to obscure the characteristic blue color. Even with the purification of diphenylamine and numerous washings of the cellular material, the green color could not be controlled in anaerobic digesting sludges[10]. The extraction and quantification of bacterial DNA was a three-stage process consisting of rupture of the bacterial cell, extraction of protein-bound DNA, hydrolysis, and evaluation of DNA content. A schematic diagram of the procedure has been included in Figure 2.

Agardy and co-workers[11] also used DNA content determinations as a digester loading parameter to relate the daily organic load applied to a biological system to some measure of the system's microorganism content. The values of DNA found in field digesters were higher than expected when compared with values reported in the literature[6] for pure cultures of bacteria (3.1 to 4.8% of the volatile solids). It was concluded that either the organism concentration during sludge digestion at detention times in excess of 20 days was higher than anticipated or interfering substances such as sugars and aldehydes were present in relatively high concentrations so as to affect the DNA determination.

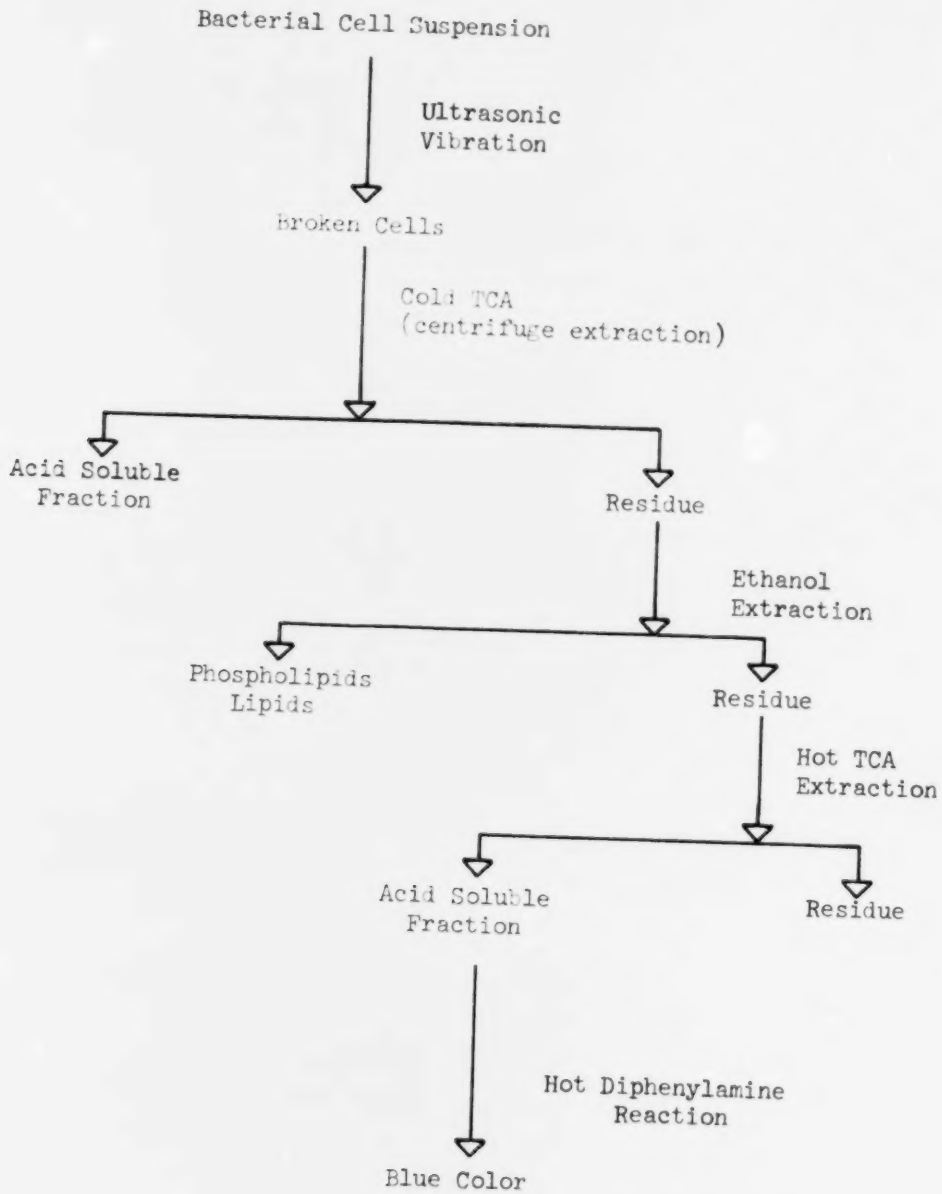


Figure 2. DNA Extraction-Analysis Procedure (9)

Fisher[12] used a modified method in analyzing digester sludge. When the DNA in the sludge samples was allowed to react with hot concentrated sulfuric acid, a yellow color resulted during hydrolysis and extraction; however, when concentrated sodium hydroxide was substituted in the first extraction-hydrolysis step, no interfering yellow color resulted. Recovery studies proved the modified DNA extraction technique to be quantitative.

Hattingh and Siebert[8,14] increased the sensitivity of the DNA method by adding an aldehyde to the diphenylamine reagent. The alcohol-ether extraction proposed by Agardy[11] was considered unnecessary. Moreover, the acid-soluble compounds of the cell debris were considered responsible for the interfering green color. DNA was liberated from the sludge with sodium lauryl sulfate by ultrasonic vibration. The supernatant liquor was collected after centrifugation and the DNA was precipitated with perchloric acid at 70°C instead of the usual boiling for 15 minutes. This method resulted in 99 and 100 percent recovery of DNA from anaerobic sludge with the addition of aldehyde to the diphenylamine reagent serving to stabilize and intensify the blue color[8].

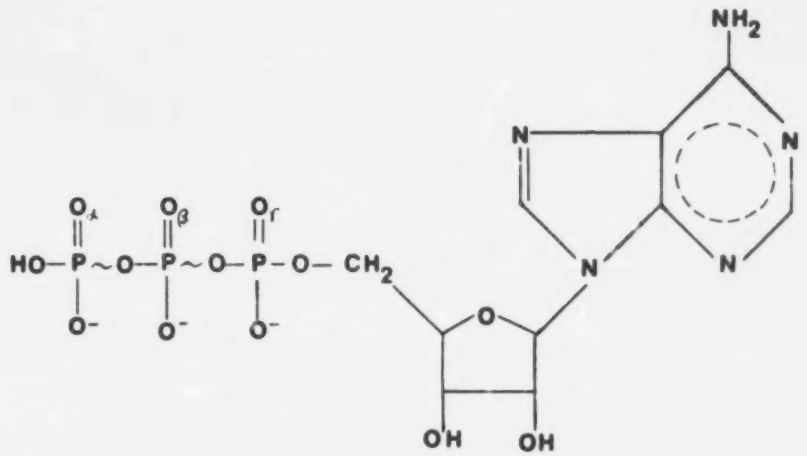
Application and Results - Genetelli[5] developed and evaluated organic loading parameters for an activated sludge system based upon DNA and the organic nitrogen used as measures of the system's organism concentration. It was concluded that a loading parameter for activated sludge may be based on pounds of BOD per day per pound of DNA in the aeration basin. A parameter based upon DNA was more universally applicable with different feed substrates than one based upon nitrogen. DNA content was considered much more sensitive to changes in sludge volume index (SVI) than solids or nitrogen concentration because it reflected the variations in bacterial concentration and the shift in type of microorganism contributing to the active biomass. The DNA content of sludge was applied as a process control parameter indicative of the type of microorganisms present in the sludge (i.e., zoogleal flocs or a more filamentous masses). The DNA content decreased prior to an increase in SVI and an abrupt change in the DNA content indicated an approaching change in the settleability of the sludge.

Holm-Hansen[14] applied DNA content to marine samples as a biomass parameter and to calculate growth rates at various oceanographic depths. A micromethod for DNA was developed based upon the measurement of the fluorescence of the complex formed by samples containing DNA incubated with diaminobenzoic acid dihydrochloride (DABA). The sensitivity of the method was in the 0.2 µg to 40 µg DNA range. To use DNA as a biomass indicator, it was considered necessary to relate DNA content of the sample to a cellular entity such as total organic carbon (TOC). DNA to TOC ratios were higher than those observed by methods such as plate count or microscopic enumeration. The data indicated that there was a considerable quantity of living material that was high in DNA and/or that the DNA was associated with particulate non-living material.

Jrgens[15] investigated the use of DNA concentrations of activated sludge as an estimate of the viable population based on the premise that the DNA released from dead cells was readily degraded by activated sludge. DNA constituted about four percent of the volatile matter of cells assuming that weight of one cell was 1×10^{-9} milligram. DNA could then be expressed as a percent of volatile solids in the sludge samples. The bacterial population of sewage, based upon DNA content, was determined to be 4.1×10^{-6} cells/ml of sewage.[15]

ATP Methodology and Application:

General Perspective - Adenosine triphosphate (ATP), a high-energy compound found in every living cell, is constantly re-formed in metabolism. It functions in a catalytic capacity acting as a link between reactions that serve as a source of energy for the organisms and those that lead to biosynthesis and growth. Therefore, as a catalyst it is conserved throughout the entire metabolic process and is a relatively constant constituent of the cell[16]. Fermentation, respiration and photosynthesis are the three major processes used by cells to extract energy from the environment and make ATP. The metabolically available energy of ATP lies in the chemical hydrolysis of the α, β, and γ-phosphate groups. The cell uses ATP to make otherwise endergonic reactions exergonic[17].



Adenosine Triphosphate

There are also some specialized energy liberating reactions, such as "firefly luminescence" that require ATP. Many comprehensive accounts on the various fundamental aspects and reaction mechanisms of bioluminescence and chemiluminescence have been published.[18-26]

Development of Analytical Methods - This literature survey will concentrate on the application of the luciferin-luciferase bioluminescence system in adenosine triphosphate measurement.

McElroy and Green[27,28] pioneered the discovery of the absolute and integral requirement of ATP for Photinus pyralis (firefly) bioluminescence and used purified luciferin and crystalline firefly luciferase to characterize the light emission reaction as being associated with the utilization of both luciferin and ATP.

Lucifer-Luciferase Reaction

$$LH_2 + ATP + E \overset{Mg^{++}}{\rightleftharpoons} (E - LH_2 - AMP) + PP_i$$

$$(E - LH_2 - AMP) + O_2 \longrightarrow (E - L - O^*) + \text{Products}$$

$$(E - L - O^*) \rightarrow (E - L - O^-) + h\nu$$

E = enzyme luciferase

LH_2 = reduced luciferin

ATP = adenosine triphosphate

Mg^{++} = magnesium ion

$E - LH_2 - AMP$ = luciferyl-adenylate complex

PP_i = phosphate inorganic

O_2 = molecular oxygen

hv = photon or quantum of light

$E - L - O^*$ = oxyluciferyl-adenylate complex, excited state

The number of light quanta emitted was determined to be directly proportional to the initial ATP concentration of the reaction system. The reaction of ATP with luciferin lead to the formation of pyrophosphate and active luciferin. The latter compound could react with either oxygen for light production or be hydrolyzed to luciferin and adenylie acid (AMP) under anaerobic conditions. Moreover, the luminescent reaction was inhibited by the end product of the reaction. Pyrophosphate liberated the enzyme from this inhibitory complex but at the same time counteracted the activation step[29]. It was noted that the light producing step was not the rate-limiting step; luciferin had to react with ATP before it could be oxidized with light production; LH_2-AMP was shown to be the active intermediate and ATP did not act as an energy donor in the luciferin-luciferase system.[20,30]

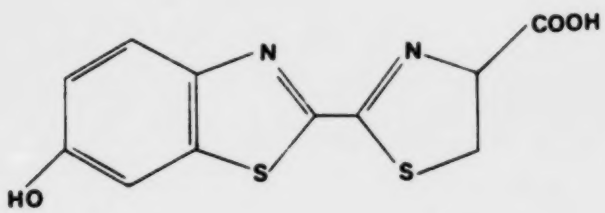
Considerable research has been performed on developing adenosine triphosphate analysis based upon the firefly luminescence system. Attention has been focused upon improving the methodology of this very complex biochemical system in the varied biological systems studied.

The basic methodology is initially concerned with complete extraction of the ATP in the sample and this primary extraction has been performed both on the reaction mixture as a whole and upon cells washed and filtered from the growth media. Extraction has been achieved with dilute perchloric acid[31-34], boiling H_2O, tris (hydroxymethyl) aminomethane (Tris) or N-tris (hydroxymethyl) Methyl-2-aminoethane sulfonic acid (TES)[35-37] trichloroacetic acid (TCA)[38], or dilute sulfuric acid [39,40]. Most recent studies have shown that in aqueous systems, the ATP of bacterial cells was quantitatively extracted with boiling (100°C) Tris buffer at pH 7.75[35,37]. Cole[31] found chilling and anaerobiosis of growing cells of E. coli before extraction with perchloric acid caused a reduction in the ATP pool. However, Knowles[41] refuted this in a study of Azobacter vinelandii where harvested, washed, and starved cells had aerobic ATP levels similar to cells taken directly from the culture medium. In general, the efficiency of ATP production was low in disrupted cells (lysis, ultrasonic rupture, or Parr bomb) compared to ATP production measurements or unbroken cell suspensions.[31,32,41]

Preparation of the lucifer-Luciferase system also has differed greatly. Lypholyzed and dessicated firefly lantern extracts should be reconstituted in a magnesium arsenate buffer which will both control the ionic strength of the medium[38] and provide the magnesium ions essential as a catalyst in the initiating step of the luciferin-luciferase bioluminescence reaction[18]. In addition, when ATP and firefly lantern extract (FLE) are combined in the presence of an arsenate buffer, an intermediate level of light emission occurs which decays steadily and exponentailly with time[19]. Some authors have preferred to use purified luciferin when analyzing submicro quantities of ATP, since in the crude, commercially available firefly extracts residual ATP and other high energy phosphate compounds[42] are found which interfere with and obscure the analysis of submicro quantities.[28]

McElroy and Green[28] observed that the light emission was modified by the ionic strength of the reaction medium, and Aledort[38] evaluated the optional ionic requirements for firefly bioluminescence. It was concluded that light emission mediated by firefly luciferin-luciferase and ATP was inhibited by increasing the ionic strength of the reaction

medium. The inhibition of firefly luciferase by ions was shown to follow certain trends, i.e., cationic inhibition occurred when $Ca^{++} > K^{+} > Na^{+} > Rb^{+} > Li^{+} > Choline^{+}$ and anionic inhibition occurred when $I^{-} > H_2PO_4^{-} > Br^{-} > ClO_3^{-} > Cl^{-} > Fl^{-} > HCO_3^{-} > COOCH_3 \cdot H_2O^{-}$.[38]



Firefly D(−) Luciferin[23]
(Active Form)

Some researchers have preferred to reconstitute the crude firefly lantern extract (FLE) and incubate it at 0-4°C from 6 to 72 hours to deplete the endogeneous luminescence associated with the residual adenosine triphosphate found in FLE[31,35-38,44]. Others have used a method of luciferin-luciferase enzyme dilution to reduce the enzyme blank due to endogeneous ATP and ATP-AMP phosphotransferase[29]. Incubating the enzyme with apyrase to eliminate endogeneous ATP and lower the blank has also been used [42] and numerous authors have observed that the enzymatic activity and luciferin content of crude firefly lantern extract varied from batch to batch thereby requiring determination of background emission levels[27], or variation of the quantity of enzyme preparation used in the reaction mixture to yield a constant for a known amount of ATP.[44]

Instrumental analysis of the photon emission by the firefly assay also has been subject to a great variety of investigations. Historically,

the recording D.C.1P121 photomultiplier tube was first used to quantify the light which was followed by a Farrand photomultiplier and the liquid nitrogen quantum counter; the ultimate sensitivity reached with these instruments was in the order of 10^{-9} g ATP/ml[19,40,45]. However, the liquid scintillation spectrometer was quickly adopted to provide sensitivities to 10^{-12} mole ATP/ml[31,46]. Many difficulties have been encountered in standardizing the methodology using this instrument due to the variabilities noted between models and type of liquid scintillation counters (LSC). Therefore, each investigator has had to adopt his particular LSC to the particular research application[29,31,37,46-48]. Such problems as whether or not to use the "in-coincidence" or "out-coincidence" mode are still a matter of controversy as is the voltage to be applied[31,37,48]. When using either a photomultiplier tube system or a liquid scintillation counter method, a standardized procedure for constant sample injection or timed reaction counting has become vital since the kinetics of the light reaction are rapid and subject to logarithmic decay. Many researchers have developed[49] unique apparatus to accomodate these variables[29,43,46] and to achieve quick sampling and injection into the reaction mixtures.[33]

Instrument companies such as Du Pont and American Instrument Company have already recently developed luminescence "biometers" based upon the luciferin-luciferase system to quantify ATP as a biomass parameter[49-51]. Defresne and Gitelman[52] developed another system employing a standard Technicon Auto-Analyzer with a Packard flow detector which in turn was connected to a Pachard Tri Carb 314F to circumvent the problems of uniform sampling and constant timing.

Application and Results - Because the amount of endogenous ATP and the ability of the organisms to further synthesize ATP were important to bacterial survival, Strange[34] investigated the effects of starvation on Aerobacter aerogenes. When washed endogenous phase organisms (10^{10}) were starved, the viability remained high (100-98%) for at least 40 hours. Although ATP was required and formed during this period of metabolism of reserves, the rate of formation of ATP decreased as the reserves declined during prolonged starvation. The content of freshly gathered and washed bacteria varied with oxygen

tension and solute concentration of the suspending solution; both anaerobic conditions and high solute concentration markedly reduced the amount of ATP extracted. The synthesis of ATP which occurred when bacteria were transferred from anaerobic to aerobic conditions or from a solution of high to one of low solute concentration was extremely rapid. Although no direct relationship seemed to exist between ATP concentration and the viability of survival propects of this bacterial population, evidence suggested that the ability of bacteria to synthesize ATP in the absence of exogenous nutrient was related to survival. Starvation progressively reduced the magnitude of the increase in ATP which occurred when bacteria were transferred from anaerobic to aerobic conditions.[34]

Forrest[39,40] studied the ATP pool in Streptoccoccus faecalis, an organism with a comparatively simple anaerobic metabolism. It appeared that a critical concentration of ATP was necessary for exponential ("log") growth to occur and at levels lower than this critical concentration, only linear growth occurred. The pool level of ATP of an organism was defined as the balance between the demands of the organism for the energy and the supply of energy derived from the catabolism of the substrate.[39]

Holm-Hansen and Hamilton[35,36] used ATP content data of the endogenous levels of ATP in laboratory cultured marine species to estimate the active biomass in ocean samples. The ATP levels found at various depths of the ocean indicated an oceanographic bacterial population of 50-2000 times greater than estimated by standard plate culturing techniques. Studying seven marine bacterial strains grown in a chemostat, the ATP content per viable cell count (standard plate count) was always high during a period of log growth and began to decline during the early endogenous phase of growth. After a short endogenous phase, all cultures studied showed a marked period during which the viable cell count decreased drastically while the ATP content in the cells decreased to a plateau level.[36]

Cole[31] examined the ATP pool in batch cultures of Escherichia coli since this organism could grow on complex or minimal media both anaerobically and aerobically with a variety of energy sources. The measurement of the ATP pool level throughout growth and starvation would indicate the extent of ATP control by the organism. The rate of ATP production was in balance with the rate of growth both aerobically and anaerobically. The ATP pool (10^{-9} moles ATP/mg dry weight of cells) remained fairly constant during "log" growth.

ATP metabolism in a strict anaerobe, Methanobacterium strain M.O.H. was investigated and the results of growth yield studies indicated that the ATP conservation was very inefficient (0.06 moles ATP/mole hydrogen) under conditions used to grow the bacterium in a fermentor[33]. In whole cell studies of this organism, ATP formation was decreased and AMP formation increased in the presence of air, chloroform, 2, 4-dinitrophenol, carbonylcyanide-m-chlorophenylhydrazone and pentachlorophenol. It was suggested that these substrates were either inhibitors of electron transport or uncouplers of an energy-linked process. The compunds also inhibited methane formation in cell-free extracts, an ATP-requiring process.

Using obligately aerobic Azobacter vinelandii[41] which possessed a very active respiratory chain system and no fermentation ATP synthesis, aerobic ATP pool levels were always high and about the same level with endogenous substrates and on anaerobiosis the ATP level fell to a quarter of the aerobic ATP pool level. D'Eustachio and Levin[50] reported the level of endogeneous ATP in bacteria to be relatively constant for several species and all phases of growth. Examining thirteen species of aerobic gram-positive and gram-negative bacteria with the aid of a "Biometer", a mean ATP content 4.7×10^{-10} µg/cell was determined. Microbial growth was related to the amount of ATP present and/or produced by metabolism of nutrient energy sources. A study of three taxonomically different organisms (Escherichia coli, Pseudomonas fluorescens, and Bacillus subtilis) during all growth phases yielded a relatively constant level of based ATP at 1.45×10^{-10} µg/cell[50] which may have been low because of sample pretreatment by sonic cell rupture[37]. This steady-state level of endogenous ATP suggested that a relatively constant level of cellular ATP

was maintained to provide sufficient energy for maintenance for vital enzyme systems during all growth phases. A linear correlation between plate count and ATP content was obtained.[50]

Kao[53], et al. reported that the rate of ATP increase was closely related to the rate of growth through lag and log phases. However, contrary to other reports, oscillatory variations were followed as the stationary phase began in pure culture studies with E. coli and P. aeruginosa. A concentration of 2 µg ATP per ml of MLSS was found most effective for the waste water having 240 mg BOD_5/l in a field study by Biospherics.[54] Excessive ATP was removed from the aeration basins by lowering sludge return rates and still maintaining the same efficiency of treatment.

Patterson[37] and co-workers developed a method for ATP analysis in activated sludge samples with relative standard deviations for replicate samples of less than two percent and ATP recovery in "spiked" samples of 98-100 percent. Results indicated the use of the ATP pool (endogeneous level of ATP in the system) as an indicator in activated sludge toxicity studies. The maximum ATP pool measured, approximately 2 µg ATP/mg of mixed liquor suspended solids (MLVSS) was typical of endogenous ATP concentration in activated sludge batch units. The maximum ATP pool occurred at pH 7.5 to 8.0 which represented normal operating conditions and was greatly reduced at low (3.0) or high (11.0) pH conditions. The effect of various concentrations of mercuric chloride on ATP levels in activated sludge after one hour incubation indicated a rapid drop in ATP pool at low Hg^{++} levels, a more gradual decrease at intermediate concentrations and increased rate of pool reduction above 10 mg Hg^{++}/liter of sludge. In studying the firefly bioluminescence analysis of ATP for toxic effects, it was concluded that any substance which inhibited luciferase activity would reduce light emission and yield false ATP results. The presence of mercuric ions reduced the luciferase activity and consequently also the light emission which effect had to be compensated for by filtration or dilution of the sample containing heavy metals. Using an endogenous ATP pool value of 2 µg/ml cell material, the pattern of the ATP pool response to changes in metabolic activity of an activated sludge

culture was determined. The results indicated a slow drop in ATP pool during a 24-hour period prior to feeding with a subsequent constant level being attained in the unfed sample during the next 24 hours as also observed by Strange and co-workers[34]. The immediate response in the fed sample was a slight drop in ATP, followed by a rapid and significant increase. The ATP pool was affected by the metabolic activity of an activated sludge culture and responded rapidly to an increase in substrate loading while being only gradually reduced as the organisms entered an endogenous phase. The study confirmed previously published reports of a relatively constant ATP pool under endogenous conditions although the pool in activated sludge was significantly lower (0.8 µg ATP/mg of volatile suspended solids) than reported for pure culture experiments (2µg per mg dry cell material). This lower ATP pool in MLVSS indicated that only a fraction of the total activated sludge solids was viable cell material. ATP was a specific indicator of cell viability since ATP content completely disappeared within 2 hours of cell death.[37]

Many applications of the luciferin-luciferase bioluminescence system have been developed. For example, the differential determination of ATP and ADP in the presence of each other is possible through the use of myokinase, phosphocreatine may be determined by the use of transphosphorifase and AMP, and glucose can be determined by the use of hexokinase and ATP by measuring the depression in luminescence[19]. Therefore, any component in a system which can be made to influence the level of ATP can be studied by this method.

A method for the estimation of ADP in ethanolic extracts of plasma was developed based upon the conversion of ATP with a pyruvate-kinase system and subsequent assay of the ATP with FLE method[43]. Studies have been conducted on membrane adenosine triphosphotase and active transport[55], the production of adenosine triphosphate in normal cells and sporulation mutants of Bacillus subtilis[45] and using ATP levels in food to indicate bacterial contamination[51].

While the FLE bioluminescence method has an absolute requirement for ATP to initiate the reaction, many other bacterial luciferases[21] catalyze the bioluminescent oxidations of reduced flavin mononucleotides in the presence of long chained aldehydes. A bioluminescent method,

$$NADH + FMN + H^+ \xrightarrow{\text{dehydrogenase}} NAD + FMNH_2$$

$$FMNH_2 + O_2 \xrightarrow[\text{long chained aldehyde}]{\text{luciferase}} h\nu$$

therefore, has been developed to directly measure the NADH oxidation in the reductive amination of α-ketoglutaric acid with NH_3[56] by means of a liquid scintillation counter and method of Stanley[47] in cell-free extracts of Nitrosomonas europaea.

Brolin[57] has further developed a photokinetic micro assay based on bacterial luciferases to include many compounds which are either convertible in dehydrogenase reactions or compounds which are involved in reactions leading to a dehydrogenase step because they can be followed by quantitating the bioluminescence produced. The bacterial luciferase may be coupled to enzyme utilizing or producing FMN or NADH to produce dynamic measurements in situ or the system may be used for measuring compounds which are conjugate in their action with NADH[57].

Dehydrogenase Activity Methodology and Application:

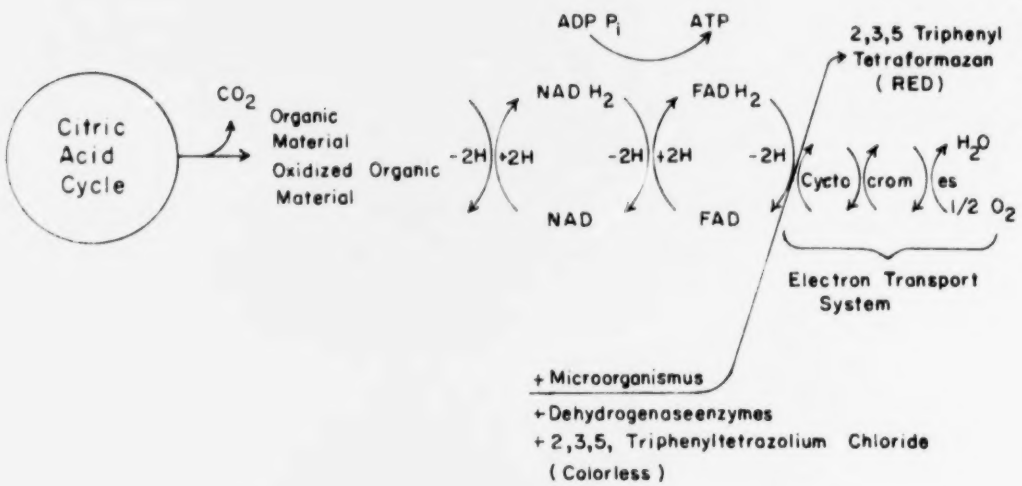
General Perspective - Attempts have been made to improve the methodology for analysing dehydrogenase enzyme activity so that it can be applied as a parameter indicative of active biomass. Various dehydrogenase enzymes or oxidoreductases are the key enzymes which catalyze the redox reactions of biological metabolism[4]. These enzymes catalyze the oxidation of organic substrate by the removal of hydrogen atoms. Every dehydrogenation must be coupled with a hydrogenation; the hydrogen atoms removed from the substrate must be added to some other compound. Many of the dehydrogenases have associated coenzymes which serve as temporary acceptors of the substrate hydrogen in the electron transport system. Two compounds which frequently serve in this capacity are the pyridine nucleotides, nicotinamide-adenine dinucleotide (DPN) and nicotinamide-adenine dinucleotide phosphate (TPN^+)[16].

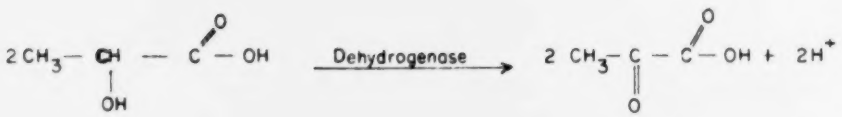
In the simplest type of enzyme catalyzed oxidation reaction, only one electron carrier is interposed between the substrate molecule and molecular

oxygen. In these dehydrogenase reactions the electron carrier is coupled to the oxygen and no other substances. However, the dehydrogenase enzyme can pass electrons to certain reducible dye-stuffs such as tetrazolium salts[1].

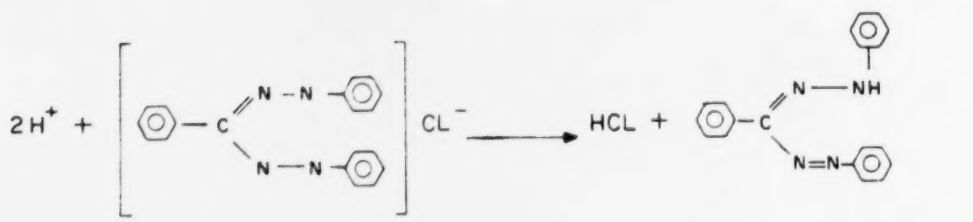
Development of Analytical Methods - Lenhard and Nourse[58,59] developed a test to measure dehydrogenase activity and applied this method to soils, benthal deposits, activated sludge systems, and anaerobic waste treatment systems. During the aerobic biological treament of organic wastes, organic carbon is oxidized to CO_2 with a concomitant reduction of O_2 to H_2O. A tetrazolium salt such as 2,3,5-triphenyltetrazolium chloride (TTC) can be employed as the hydrogen acceptor during this dehydrogenase-catalyzed oxidation reaction. The hydrogen released during the reaction is attached to the salt yielding the highly red-colored redcued compound, triphenyltetraformazan (TF) which is easily extracted into alcohol and measured spectrophotometrically according to Beer's Law. (See Figure 3)

Lenhard and Nourse[58,59] also investigated the effects of pH, temperature and reaction time during analysis. The optimum pH was 8.4 for samples obtained from anaerobic and aerobic systems; strongly alkaline pH values resulted in possible tetraformazan production. The optimum temperature for the incubation of the tetrazolium chloride with activated sludge and anerobic digestion samples was 37°C. Batch studies indicated an increase in dehydrogenase activity within one hour after substrate addition and continuous culture (extended aeration) studies indicated that the dehydrogenase test offered the possibility of measuring the availability of endogenous substrates by comparing the reduction of TTC with and without added glucose. In the absence of glucose, low values of dehydrogenase activity would indicate a low concentration of endogenous metabolites available for oxidation. Without siginficant modification of the analysis, the dehydrogenase activity in anaerobic samples was tested both in the presence of air and in vacuo; the in vacuo test results were slightly higher but the difference was not considered significant enough to warrant the further complication of the method. The rapidity of the dehydrogenase test (1 hour), allowed for the detection of impending process difficulties before any change in the quality of effluent could be measured.





Substrate Enzyme Oxidized Substrate



2,3,5 Triphenyltetrazolium Chloride (Colorless)

2,3,5 Triphenyltetraformazan (Red)

Figure 3. Transfer Mechanisms of Intermediate Substrate Metabolism

Bucksteeg[60] expanded Lenhard's work to include the evaluation of a prototype activated sludge plant and improved the methodology to include the exclusion of light during the incubation period which he extended to one hour to eliminate the photochemical effect. These investigations indicated that the overall activity of dehydrogenases in a wastewater and activated sludge mixture increased with increasing concentrations of the dry mass of sludge, whereas the biochemical efficiency of the sludge relative to unit weight of dry mass decreased.

Ford[1] further investigated the dehydrogenase activity reaction time for samples obtained from a contact stabilization process and determined that the production of tetraformazan declined rapidly after 60 minutes and was a direct function fo sludge age. The TTC-measured dehydrogenase activity responded to significant changes in plant loading. Jones[61] examined the effect of extended aeration on the dehydrogenase activity. Oxygen was demonstrated to be a competitive inhibitor in TTC→TF reduction. The reaction reached the same level of reduction aerobically as it did anaerobically but it took longer to attain that level (characteristic of competitive inhibition). It was concluded that the dehydrogenase test more accurately measured the total biological activity of an anerobic system since molecular oxygen acted as an inhibitor and competed with TTC as a terminal hydrogen receptor.

Shih and Stack[62] used the dehydrogenase test to explore the temperature effects on energy-oxygen requirements in an aerobic biological oxidation process. Energy-oxygen was defined as the net consumption of oxygen in support of synthesis reactions. The methodology was refined during the studies to include the extraction and concentration of TF with 1-butanol because all the TF produced (including that associated with the solids) was concentrated in the limited butanol system. To preclude a possible TF production interference by dissolved oxygen, it was removed with sodium sulfite prior to the addition of TTC. The energy-oxygen coefficient developed during the studies varied with substrate oxidized and temperature conditions.

Marlar[63] noted the inhibitory effect of oxygen on the dehydrogenase test and by scrubbing the samples with nitrogen, increased the color intensities and the reproducibility of the reduced and extracted TF. Ghosh[64] included initial cell disruption and a one-hour sample incubation at 37°C with TTC under a nitrogen atmosphere to eliminate the effects of dissolved oxygen and microbial growth during the test period. The dehydrogenase activity was proportional to the solids concentration and the constant of proportionality was independent of growth rates for a given microbial population and substrate composition.

Dean and Rodgers[65] investigated the steady-state levels of dehydrogenases of Aerobacter aerogenes in a variety of nutrient-limiting chemostats at various dilution rates between 0.1 and 1.0 hr^{-1}. These dehydrogenase activities were determined by the TTC reduction method and it was shown that the dehydrogenase activities were generally higher when sugars provided the carbon for growth than in other nutrient-limiting conditions. Randall[66] investigated the use of dehydrogenase activity as a predictive parameter for activated sludge drainability. Results indicated that a dehydrogenase activity to solids ratio of less than 0.6 μmoles per gram of solids was assurance of good sludge drainability.

SECTION IV

INTRODUCTION

This research was initiated to define the applicability and limitations of the dehydrogenase test for the measurement of active biomass content of biological sludges used in the treatment of domestic and industrial wastewaters. During the studies reported herein, experimental apparatus were constructed, analytical techniques established, and pure and heterogeneous batch culture and continuous culture studies with and without sludge recycling were conducted.

Experimental Apparatus:

Pure Culture Studies - In this phase of the experimental studies a pure culture of E. coli was grown in a minimal substrate (Table 1). The ractor system was designed to permit aseptic techniques and to eliminate possible external contamination. To accomodate this requirement, three double sidearm, water-jacketed one-liter Spinner flasks were arranged as shown in Figure 4. Both sidearms of each flask were sealed with serum stoppers and hypodermic needles were passed through them to allow for both gaseous interchange and for feeding and sampling. The compressed air used for aeration was filtered through a Gelman filter holder containing a 0.45μ, 25 mm diameter filter paper prior to its introduction and diffusion into the culture medium.

Inside the flask reservoir, a glass air diffuser was attached with tygon tubing to the needle extending through the serum stopper to provide for aeration below the substrate level in the reactor. An air outlet port was provided by attaching a cotton-plugged syringe barrel to a hypodermic needle inserted through the stopper next to the air inlet. The sampling port consisted of a similar arrangement including a length of tygon tubing attached to the needle and extending well below the surface of the substrate in the reactor. The cotton filled syringe could be removed aseptically from the needle and either a filled or an empty sterile syringe could be attached for feeding or sampling respectively.

TABLE 1

Minimal Substrate* for Batch Cultures

Nutrients	Concentration of Stock Solutions	Volume of Stock Solution Used in the Reactor	Final Concentration in the Reactor
$(NH_4)_2SO_4$	18.857 g/l	As selected	to maintain C:N of 20:1
Substrate	As selected	As selected	
$MgCl_2 \cdot H_2O$	32 g/l	5 ml	160 mg/l
$CaCl_3 \cdot 2H_2O$	400 mg/l	5 ml	2 mg/l
$FeCl_3 \cdot 6H_2O$	400 mg/l	5 ml	2 mg/l
KH_2PO_4	5.4436 g/l (0.04M)		Use equimolar volumes of both and bring final volume up 1 liter, which yields:
Na_2HPO_4	5.6784 g/l (0.04M)		pH 6.88 and 0.08M $PO_4^{=}$ concentration

* Minimal Substrate: a simple synthetic medium consisting of ammonium salts, phosphates, sulfates, and other mineral salts with the addition of organic compound as a source of carbon and energy.

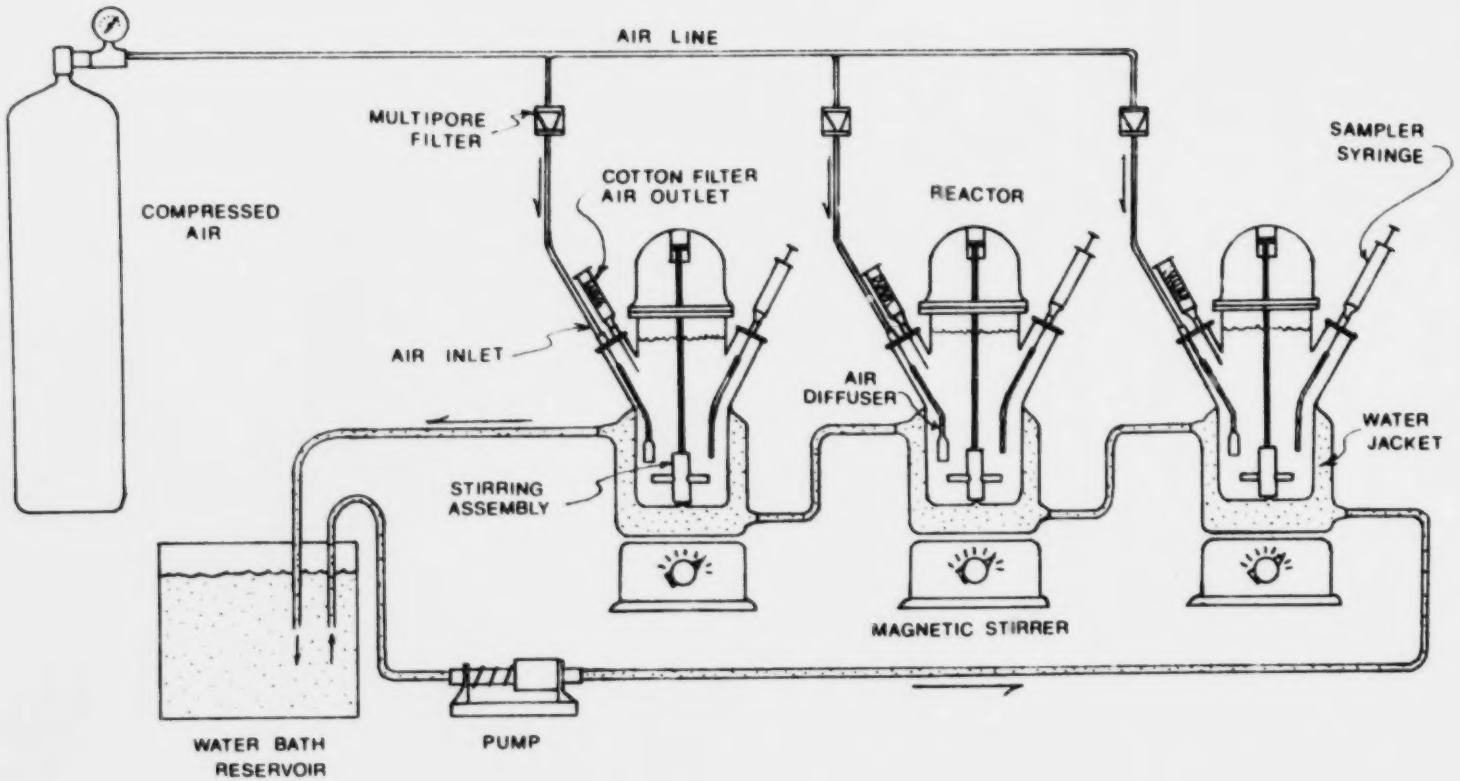


Figure 4. Reactor Assembly for Pure Culture Studies

A magnetic stirrer was placed beneath each reactor to provide internal mixing by rotation of the magnetic impeller inside the flasks. A piece of asbestos was placed between each reactor and the stirring apparatus to control heat transfer. The temperature in the reactors was maintained at 37°C by circulating water from a water bath through the water jackets surrounding each culture flask.

Prior to each experiement, one liter of substrate was measured into the reactor flask and sealed with a dome-shaped top containing an inner sleeve to align the impeller assembly in the center of the substrate reservoir. The top was secured by a heavy wire flange clamp and the complete assembly was then autoclaved at 15 psi and 120°C for 15 minutes.

Heterogeneous Culture Studies - The 10-liter volume reactor unit is schematically shown in Figure 5. The essential elements are the reactor in which biological growth occurs, a mixing motor and propeller, an air diffuser located in the bottom of the reactor, a variable speed influent pump, and an electronic level controller. Compressed air was supplied throughout the aerobic batch and continuous culture studies. The air supply was regulated by Air Flowmeter (SHO-Rate, Model 1355, Brooks Instrument Div., Emerson Electric Co.).

During the batch culture studies, nutrient media similar to that used during the pure culture studies were seeded and pumped into the reactor followed by a predetermined amount of selected substrate. The mixing units consisted of a B & B Motor (1725 rpm, 115 V, 1/8 HP, NSH 54 type) and controller (B & B Motor and Control Corp., N.Y., N.Y.) and provided complete mixing along with air diffusion. The reactor temperature was maintained at 20°C during these studies.

In the continuous culture study, both nutrient media and substrate were stored in a reservoir and pumped into the reactor at a selected flow rate to give a desired retention time. During the anaerobic digestion study, however, the air supply line was sealed off and the level controller was employed to regulate the reactor volume. An effluent pump (Model 7015, Master Flex Pump, 500 rpm, 115 V. Cole-

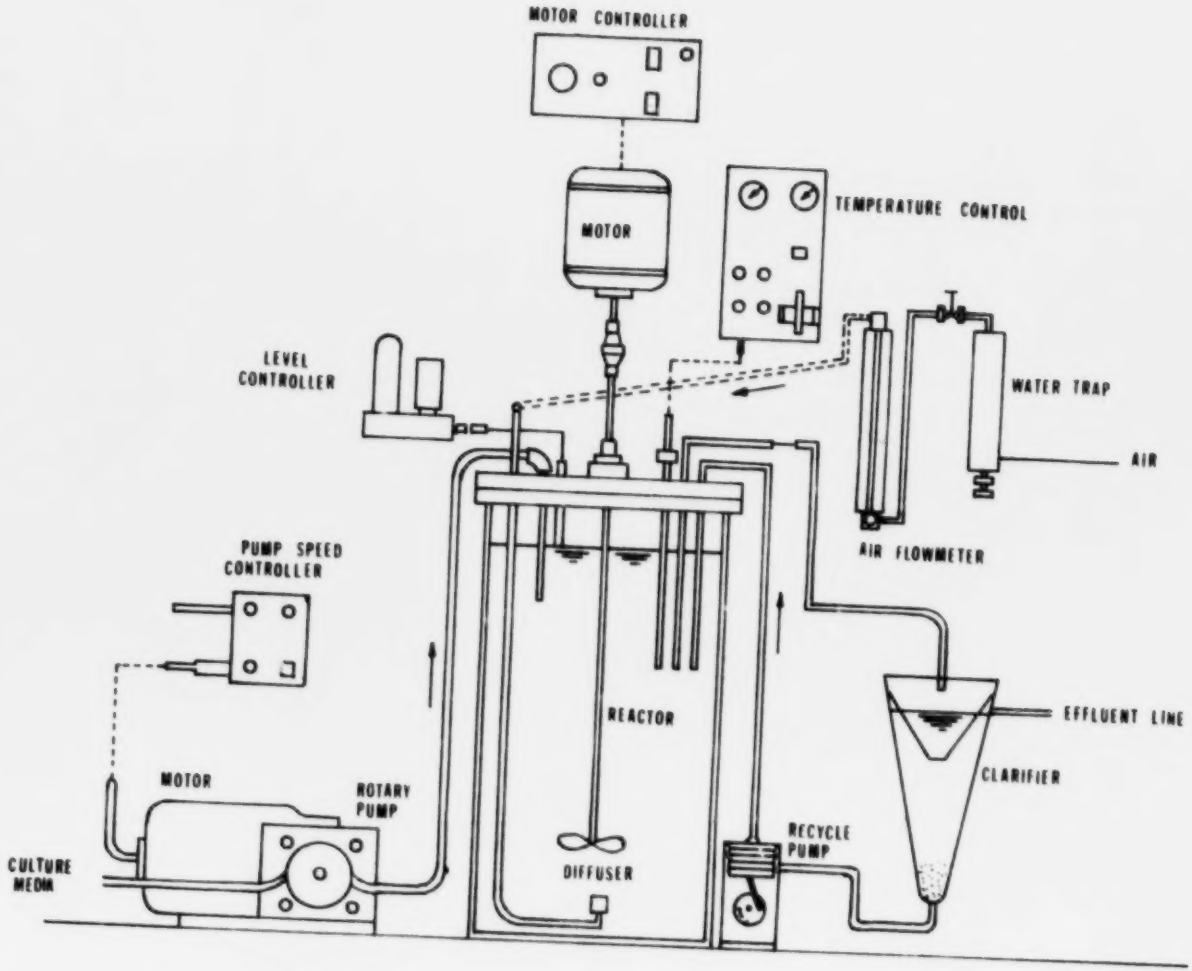


Figure 5. Reactor Assembly for Heterogeneous Culture Studies With Solids Recycle

Parmer Instrument Co., Chicago, Ill.) was connected to the effluent line and either activated or deactivated by the level controller as soon as the reactor contents went above or fell below the 10-liter mark, respectively. A temperature of 37°C was maintained by exterior heating tape and a heating element inside the reactor controlled by a temperature controller. During the sludge recycle studies, a 2.7-liter clarifier and recycle pump (115 V, 7.5 rpm. Gorman-Rupp Corp., Bellville, Ohio) were connected to the reactor system as shown on Figure 5. The biological seed was obtained from the activated sludge process and anaerobic digester of the South River Water Pollution Control Plant in Atlanta, Georgia for the aerobic and anaerobic studies respectively.

Pure Culture Preparation:

The test organism for the pure culture studies, E. coli was cultured for 18 hours on a nutrient agar slant and then washed from the slant with three separate applications of distilled water, concentrating the cells by centrifugation after washing. The washed cells were resuspended in sterile water and a known volume of E. coli suspension was used to innoculate each reactor. Nutrient agar plates were also streaked at this time to determine the absence of contaimination.

In some of the batches, growth and substrate were monitored from the time of initial innoculation whereas in other cases, the population was allowed to acclimate to the substrate over night (about 16 hours) after which time additional substrate was added. The analyses were then followed from the beginning of substrate removal thereby avoiding the lag period observed when the population was first acclimating to the culture medium. In either case, log growth was easily observed.

Analytical Techniques:

Each batch conducted during this phase was monitored by standard plate counts, Coulter Counter enumeration, substrate removal, total suspended solids determination, dehydrogenase activity and, on occa-

sion, adenosine triphosphate (ATP) content. The analytical determinations required a minimum sample volume of 30 ml and at least 30 minutes between samplings. The various tests could be performed concurrently and some samples could be stored and frozen for later analysis. When substrate volumes were reduced below 400 ml by sampling, the culture medium was diluted back to one liter by addition of sterile phosphate buffer solution. Usually one day was required to prepare and sterilize the reactors, innoculate and grown the cultures, and to prepare the necessary reagents and sampling vials before a batch study could be initiated.

Coliform Analysis - The Millipore Filter plate count technique for the analysis of coliform as described in Standard Methods for the Examination of Water and Wastewater (Standard Methods)[67] was employed in these investigations for E. coli. A 1.0 ml sample of the proper dilution was filtered and rinsed through a stainless steel Hydrosol filter holder and the bacteria were retained on a 0.45μ pore size, 47 mm diameter, sterile Millipore filter. The filter was then placed onto a sterile pad soaked in 2 ml of MF Endo Broth in a sterile petri-dish, inverted and incubated at 37°C for 18 to 24 hours. The coliform analysis was initiated as soon after the sample was drawn in order to prevent growth or culture attenuation. The filter holder was maintained in a sterile condition by exposure to ultraviolet light for at least one minute before each filtration[68]. Results were generally available within 24 hours of sampling.

Solids Determination - Total suspended solids were determined gravimetrically on 0.45μ, white grid, 47 mm Millipore filters. The filters were individually washed with distilled water under vacum for approximately two minutes in order to remove the glycerine and wetting agent and to insure a constant tare weight. Each filter was numbered, dried for 30 minutes at 103°C and desiccated for at least 30 minutes before weighing. Filters were also stored in the desiccator prior to use.

An exact volume of sample was vacum filtered through the Millipore filter and the filtered solids (cells) were dried with the filter at 103°C for 30 minutes, desiccated for at least 30 minutes, and then weighed. The tare weight was subtracted from the final weight obtained

and the difference was mutiplied by the volume factor of that sample thereby giving the results in mg/l. The samples were analyzed for solids shortly after removal from the reactors to prevent settling, clumping and possible changes in bacterial populations. The very low populations (approximately 10^7 organisms/ml) and the limited sample volume (10-20 ml) characteristic of the pure culture batch studies required considerable care in analysis since samples often yielded less than a 10 mg weight change. Later solids analyses were improved (Batches 7-10) by using a double filter-tare method (Appendix A).

Determination of Substrate Concentrations - Various methods of substrate determination were employed depending upon substrate and technique available. Glucose and galactose concentrations were monitored by "Glucostat"[69] and "Galactostat"[70] assay techniques which measured the substrate concentration spectrophotometrically. Standard glucose or galactose solutions of known concentrations were determined with each set of samples analyzed in order to circumvent the problem that slight changes in the incubation times of the sample with the test reagent produced variations in color intensities even with identical glucose or galactose concentrations. In addition, all samples were filtered through a glass fiber filter to rid them of bacterial solids which would otherwise assimilate glucose or galactose during the test period and also contribute turbidity interferences during absorbance measurements. The glucose samples were incubated with the test reagents for 30 minutes and the color developed was stable for at least 12 hours thereby permitting delayed Beckman DU analysis. The galactose samples required much more care in analysis since the color development during the incubation period frequently was poor and unstable so that samples could not be stored more than 3 hours.

Chemical oxygen demand (COD) determinations provided a measure of the oxygen equivalent of that portion of organic matter in the sample subject to oxidation. In monitoring sucrose in pure culture studies with E. coli, the COD assay was employed according to Standard Methods. All samples were filtered through glass fiber filters to rid them of bacterial solids which were also susceptible to strong oxidation.

The samples were refluxed with known amounts of potassium dichromate and sulfuric acid, and the excess dichromate was titrated with ferrous ammonium sulfate. The amount of oxidizable organic matter measured as oxygen equivalent was proportional to the potassium dichromate consumed.

The Beckman Total Carbon Analyzer was used to monitor the acetic acid and L-alanine substrates in pure culture studies (Batches 8 and 10). All samples were filtered through Millipore filters (0.45 μ) and preserved with HCl to prevent further growth before analysis. When the sample was injected into the Total Carbon Analyzer, it was completely combusted to CO_2 and the CO_2 was analyzed in the instrument by infrared spectroscopy. Appropriate standards were analyzed and treated in the same manner for calibration purposes.

Gas-liquid chromatography was also employed to follow the acetic acid uptake (pure culture Batches 8 and 9). A model 700 F&M gas chromatograph with hydrogen flame ionization detector and six feet, 1/8 inch-diameter stainless steel columns packed with 20% carbowax 4000 and TPA on 60 to 80 mesh WAWDMCA (high performance chromosorb W, acid washed, silanized) was used. All samples were filtered through Millipre filters (0.45 μ) and preserved with HCl to prevent further growth. Retention time was five minutes using this column packing with excellent resolution.

L-alanine in Batch 10 was monitored on both the Beckman Total Carbon Analyzer and the Technicon Auto Analyzer for ammonia and total nitrogen. Based upon the calculated value of the total nitrogen available in the standard solution of pure L-alanine (1200 mg L-alanine or 192 mg nitrogen), the results obtained from the Auto Analyzer indicated that apporximately one-half (100 mg) of the total nitrogen content of L-alanine was available as ammonia. The possible explanations for this inconsistency were (1) the amino group of L-alanine was hydroloyzed upon standing in aqueous solution, (2) the pure L-alanine crystals were contaminated with ammonia salts, or (3) the amino group was cleaved from L-alanine when coming into contact with the strong digestion mixture (H_2SO_4, $HClO_4$, and catalyst) used in the auto-analysis procedure.

Coulter Counter Analysis - A methodology for employing the Coulter Counter for correlation of bacterial populations with plate counts was developed in accordance with the experiences of Swanton[71]. By diluting a known volume of sample in Isoton (particle-free saline), the bacterial population at the time of sampling was fixed thereby allowing delayed analysis. A 1.0 ml sample was most frequently used to provide an adequate counting range; however, on occasion, the sample required further dilution when the organism concentration exceeded 10^7 organisms per ml.

To eliminate the interference of very high background noise, a metal wire mesh "cage" was positioned around the sampling namometer and aperture tube stand of the counter. The instrument was calibrated with 3.49 μ diameter latex spheres and windowed to count all particles between 3-5 μ diameter. A 50 μ aperture tube was employed and a 0.05 ml sample aliquot was counted. To obtain a recordable population count, each sample was counted five times, the total count averaged, corrected for background and coincidence, and multiplied by the appropriate dilution factor. In later batches (7-10), a multiplication factor of 100 was used for data interpretation.

Adenosine Triphosphate (ATP) Analysis - The first method employed for ATP analysis was patterned after the work of Patterson et al.[37] This method entailed the preparation of a luciferin-luciferase enzyme reagent and ATP standard solutions, ATP extraction from the samples, and determination of ATP content in the samples by counting the light emissions with a Packard Tri Carb Liquid Scintillation Counter (Model 3320).

The luciferin-luciferase enzyme preparation required dissolving one vial of Sigma desiccated firefly tails in 37.5 ml of deionized distilled water and allowing it to stand at room temperature for one hour. The solution was then filtered through a Watman #3 filter and then allowed to incubate in an ice bath for 24 hours. The ATP standards were prepared in 0.025 M tris buffer to desired concentrations.

For sample analysis, 2.0 ml of sample were transferred to 50 ml NPN tubes containing approximately 40 ml of boiling tris buffer (0.025 M, pH 7.75) and held in boiling water for 10 minutes with occasional shaking

to kill the bacterial population and extract the ATP quantitatively. The tubes were then rapidly colled and brought to volume with additional tris buffer. For an immediate assay, the tube was then placed in an ice bath; for later analysis, the samples were frozen at -20°C.

The scintillation counter was set with a gain at 100 percent amplification, a window setting of 50-1000 on the "Red Channel", and the coincidence mode was switched to off. Counts were taken for 0.1 min (6.0 seconds) and background from the luciferin-luciferase preparation was measured prior to each analysis. Samples were inserted into the counter by transferring 1.5 ml of enzyme preparation into a scintillation vial followed by 0.5 ml of ATP sample (or standard) and mixing. Since the luminescence decayed exponentially with time, the interval between addition of the ATP to the enzyme preparation and the initiation of the counting sequence was carefully controlled. The data was analyzed graphically due to the random variability of 6-second counts, extrapolating the line of best fit back to one minute since exponential decay commonly began one minute after the reaction was initiated. The graphical technique provided linear standard curves for a wide range of ATP concentrations. Attempts to apply this methodology to pure culture batch studies during the investigations have proven inconclusive and have further emphasized the sensitive nature of the analytical procedures particularly where relatively low biomass concentrations were developed. This sensitivity can be ascribed to the necessity of control of physical parameters during analysis. For example, the temperature at which ATP extraction from the cells takes place has been found to be very critical and a few degrees of temperature fluctuation will greatly change the ATP yield[31,37]. Moreover, the pH and the volume of both the sample being extracted and the sample aliquot being measured in the liquid scintillation counter are very critical[48]. Exact and consistent timing of all steps in the procedure is essential[29].

Considerable difficulty has been experienced in standardizing these variables and generating reliable and reproducible data. Adaptation of the liquid scintillation counter became questionable primarily because of the relatively low organism populations and the necessity for switching

the counter out of coincidence mode of scaling due to the danger of "stripping" the photomultiplier tubes when high intensity light is emitted. The possibility of reducing the light intensity through both enzyme dilution and aging, and decreasing the volume of sample aliquot being counted were investigated without success. As a consequence, this method was abandoned at this point.

The second method employed for ATP analysis was as proposed by McElroy, et al.[30] This method entailed the preparation of a luciferin-luciferase enzyme reagent and ATP content photometrically through conversion of the light intensity and its proportional transfer to a digital readout unit. The instrument was calibrated for each reaction mixture so that the ATP concentration was read directly. The luciferin-luciferase enzyme was supplied with a buffer salt in tablet form. After dissolving one tablet of buffer salt in 3.0 ml of ATP-free Low Response Water (acidified, boiled, neutralized to pH 7 with NaOH and autoclaved distilled water), one vial of enzyme substrate was added and 0.1 ml transferred into each reaction cuvette with an automatic pipettor.

In preparing the ATP standard, 100 ml of fresh 0.01 M morpholinopropane sulfonic acid (MOPS) buffer and 100 mg of crystalline adenosine-5' - triphosphate-disodium salt were mixed to make a stock solution. From this solution, serial 1:100 or 10:100 dulutions with 0.01 M MOPS were made until the final ATP concentration was 0.1 μg ATP/ml or 1×10^8 fg (femtogram) ATP/ml. This final solution was dispensed in about 0.5 ml aliquots into clean cuvettes, capped, frozen and stored. The frozen standard was thawed, brought to room temperature and injected into the reaction mixture when the samples were ready for analysis.

The ATP extraction method for the samples by Dimethysulfoxide (DMSO) preceded by freezing and thawing was chosen as recommended by DuPont. The extraction procedure is included in Appendix B. A comparison of the ATP extraction method by DMSO and by nitrogen bombing was also accomplished in an attempt to shorten and simplify sample preparation as indicated in Table 2 and Figure 6 for data obtained from selected batch and continuous culture studies. In the nitrogen bombing method, the samples were treated in a Parr Bomb by exposing at 30 atmospheric

TABLE 2

Comparison of ATP Contents Extracted by DMSO and by Nitrogen Bombing

unit: fg/ml

DMSO	Bombing	Sources	DMSO	Bombing	Sources
6.78×10^7	1.81×10^7	Aerobic, continuous	5.60×10^7	1.25×10^7	Aerobic continuous cultures
8.56×10^7	2.10×10^7	Cultures grown on	3.60×10^7	3.75×10^6	Grown on chicken processing
1.46×10^8	3.09×10^7	glucose.	8.82×10^6	2.14×10^5	wastes.
8.23×10^7	1.47×10^7	" "	5.22×10^7	2.78×10^6	" "
1.03×10^7	3.84×10^6	" "	3.80×10^7	5.41×10^6	" "
$1.9. \times 10^7$	5.67×10^6	" "	9.20×10^6	8.20×10^6	Aerobic continuous cultures
1.31×10^7	4.47×10^6	" "	3.60×10^7	1.00×10^7	grown on fish processing
3.46×10^7	1.50×10^6	Anaerobic batch	2.60×10^7	4.10×10^6	wastes.
1.56×10^7	1.03×10^6	cultures grown on	4.80×10^7	7.40×10^6	" "
1.27×10^7	7.10×10^5	leachate.	1.70×10^7	5.13×10^6	" "
9.60×10^6	1.22×10^6	" "	1.55×10^7	2.02×10^6	Aerobic batch cultures
8.46×10^6	1.64×10^6	" "	1.93×10^7	2.80×10^6	grown on acetic acid.
8.52×10^6	5.03×10^5	" "	2.33×10^8	7.17×10^7	" "
9.48×10^6	1.13×10^6	" "	1.36×10^8	3.63×10^7	" "
7.26×10^6	6.29×10^5	" "	8.94×10^7	2.96×10^7	" "
7.56×10^6	8.56×10^6	" "	2.94×10^7	4.61×10^6	" "

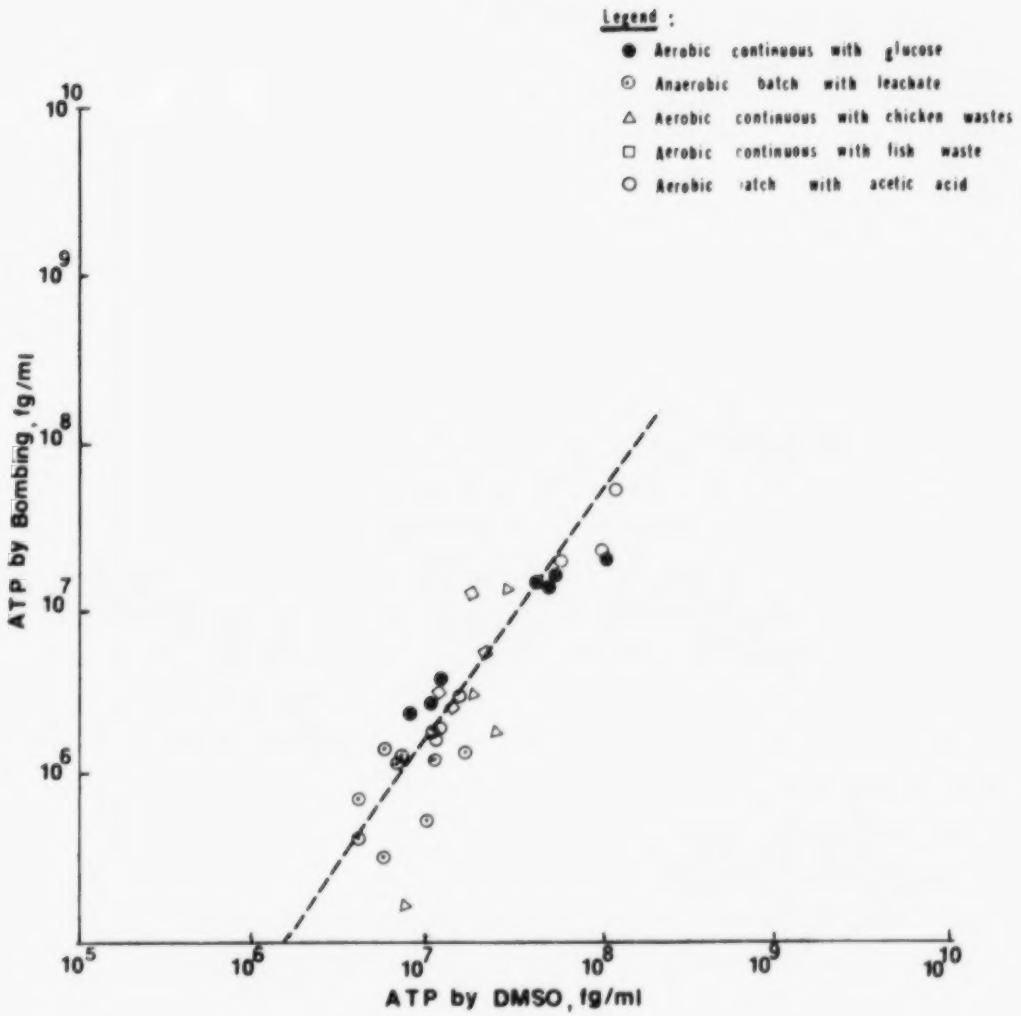


Figure 6. Comparison of ATP Contents Extracted by DMSO and by Nitrogen Bombing

pressure under nitrogen and stored in the freezer until ready for Biometer analysis.

As shown in Figure 6, there was no definite numerical relationship observed between the amount of ATP extracted by DMSO and by nitrogen bombing, even though general similarity was shown over the indicated ranges of concentrations. The nitrogen bombing technique did not show as much reliability or reproducibility as the DMSO extraction method. The amounts of ATP extracted by DMSO preceded by freezing (Table 3) yielded the highest values, while those by nitrogen bombing with freezing resulted in increased concentrations but much lower than those observed by DMSO extraction. As a consequence, the DMSO extraction method was employed for the ATP analysis throughout present studies. However, the continuous culture studies indicated definite relationships between the two methods in certain retention time ranges; i.e., ATP concentration by bombing vs. by DMSO was close to 90 percent at 20 hours and 30 percent near 12 hours or shorter retention times. Under consistent operation schemes as those used during activated sludge treatment or other biological processes in the field, this relationship would possibly be constant and the bombing method could therefore give satisfactory results. This consistency is demonstrated on Figure 6 where the higher magnitude correlations were obtained at longer retention times for the continuous culture study with glucose. Additional effort must be directed toward definition and/or development of a possible correlation between the two methods of ATP extraction of samples from aerobic and anaerobic processes during the subsequent field studies.

Dehydrogenase Analysis - Numerous investigators have attempted to develop a standardized method for the measurement of dehydrogenase as an indication of active biomass. Most work has been performed with heterogeneous continuous culture studies or pilot plant studies of activated sludge systems. However, these studies were limited to systems with high microbial populations as stimulated by non-limiting substrate levels. The study reported herein emphasized application with pure and heterogeneous cultures with various selected substrates.

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TABLE 3

Effects of Freezing and Nitrogen Bombing on ATP Contents Extracted

	Nitrogen Bombing fg/ml	DMSO Extraction fg/ml
Before Freezing	1.18×10^6	1.06×10^8
	1.07×10^6	1.14×10^8
After Freezing	1.82×10^7	1.23×10^8
After Refreezing	2.03×10^7	8.90×10^7

The method adopted for these investigations was proposed by Ghosh[64] and included initial cell disruption and incubation of the sample with 2,3,5-triphenyltetrazolium chloride under a nitrogen atmosphere to eliminate the effects of dissolved oxygen and microbial growth during the test period. The inhibitory effect of oxygen on this method was first observed in these laboratories by Marlar[63], when nitrogen was bubbled through the test samples, the color intensities of the tetraformazan became highly reproducible. The apparatus used in these investigations is illustrated in Figure 7 and the analytical method is detailed in Appendix C.

To properly interpret the results with respect to dehydrogenase activity, it was necessary to be able to express a relationship between dehydrogenase activity and biomass concentration which would permit direct conversion of the analyses for dehydrogenase activity obtained during these studies to an expression of active biomass. Since the pure culture studies of this phase of the investigation utilized substrates similar to those reported by Ghosh[62], the empirical equation developed by this author was also employed in these studies in accordance with the following:

$$X = 4.4 + 536\,A \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

where:

X = biomass concentration, mg/l

A = dehydrogenase activity

Equation 1 was developed by multiple regression analysis of data from a series of soluble substrate studies utilizing glucose and galactose in which the biomass concentration was plotted against the dehydrogenase activity as absorbance at 1.0 cm light path and 483 mμ wavelength. These data and the resulting equation of the curve of best fit are shown on Figure 8 together with the data obtained for substrates used during these studies.

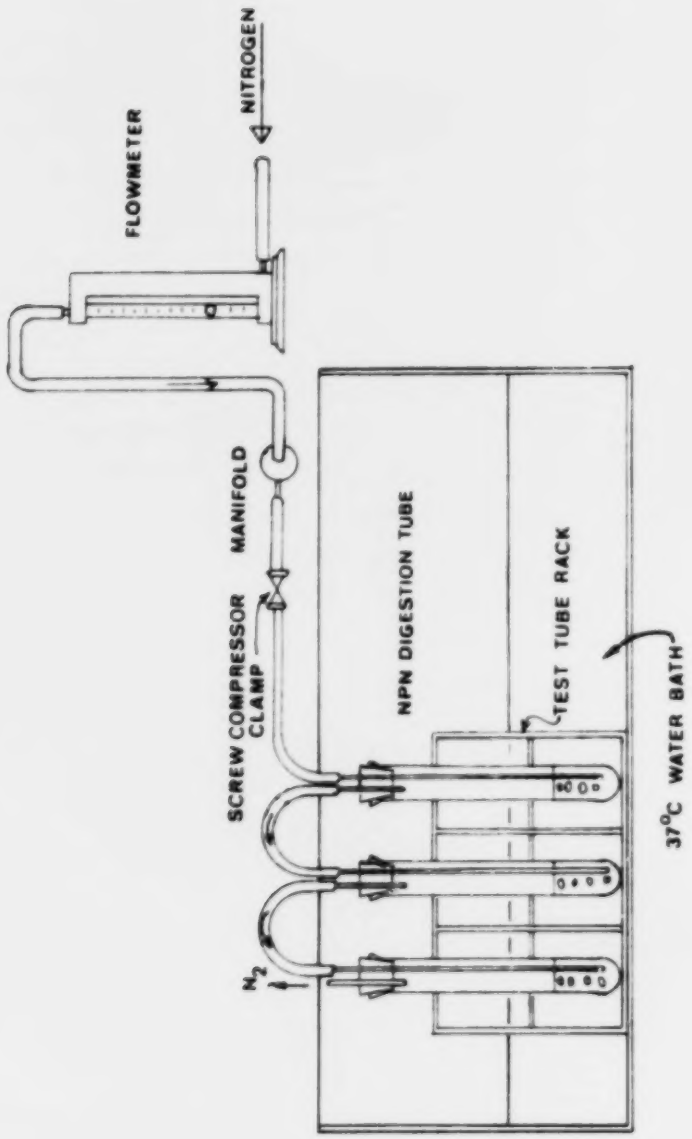

Figure 7. Apparatus for the Dehydrogenase Test

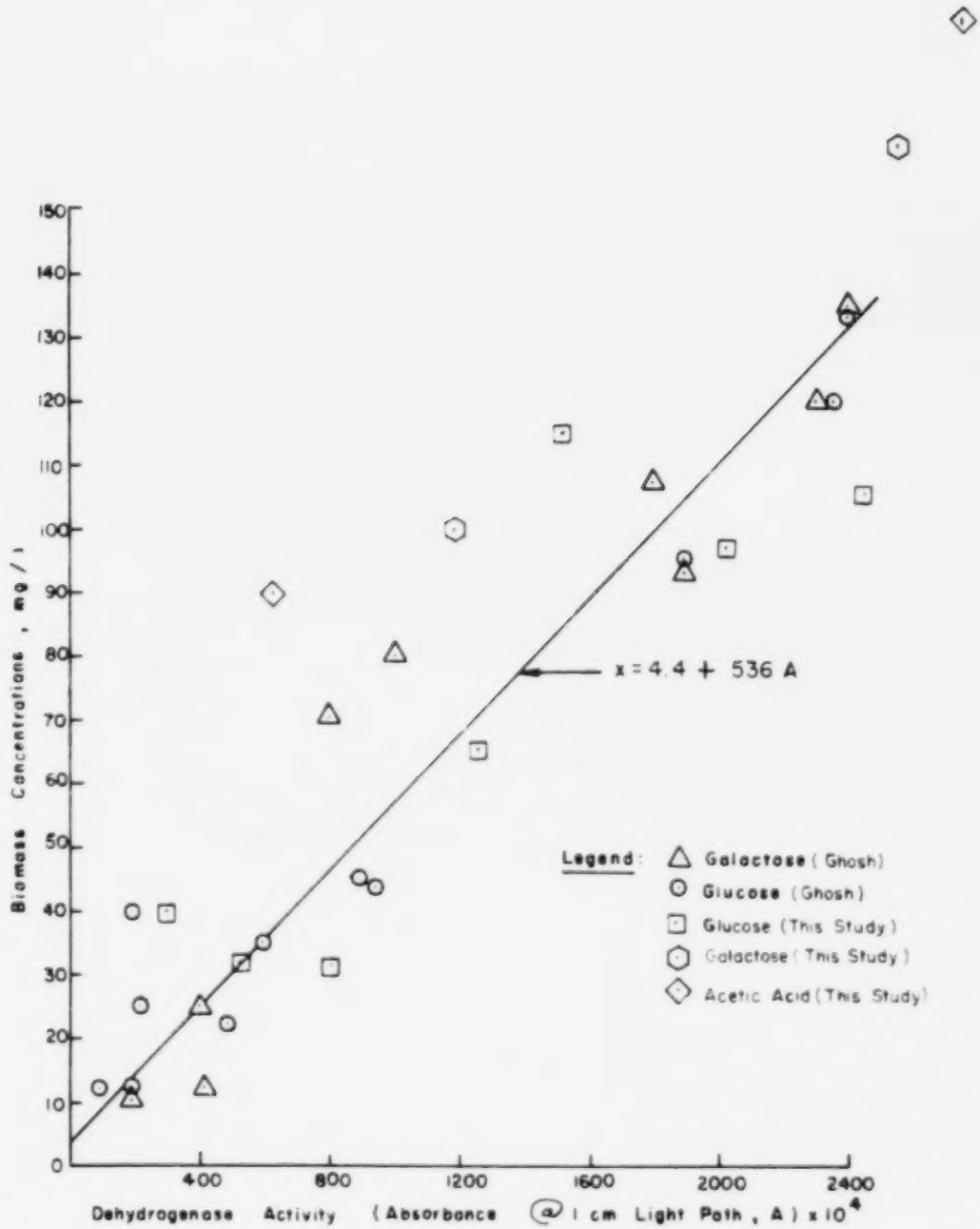

Figure 8. Correlation Between Dehydrogenase Activity and Biomass Concentrations

One of the difficulties experienced with the dehydrogenase measurement was that when the samples were homogenized in Waring Blender, little color was developed and large analystical variations existed between duplicate sampels. Moreover, since the untreated samples had more color than the homogenized samples, the use of the blender was discontinued and a cell disruption technique[72] was developed to eliminate this variance and insure consistency of analysis.

In this method, the samples were treated in a Parr Bomb by exposure at 30 atmosphers of nitrogen. Better color development was experienced than with the untreated or homogenized samples and little significant deviation was noted for duplicate samples, Moreover, as indicated in Table 4, there was good correlation between the dehydrogenase activity of samples analyzed immediately after distintegration in the Parr Bomb and those frozen after treatment in the Bomb and analyzed after thawing. This latter observations permitted storage of samples for subsequent analysis in number.

TABLE 4

Comparison of Sample Pretreatment Method for Dehydrogenase Analysis

Sample Number	Untreated Sample		Homogenized Sample (a)		Disintegrated Sample (b)		Disintegrated & Frozen Sample (c)	
	A (d)	X (mg/l)	A	X	A	X	A	X
1A	0.1805	99.9 (e)	0.0339	20.9				
1B	0.2381	135.2	0.0283	17.7				
1C	0.1221	66.3	0.0232	14.9				
2A	0.0883	51.2			0.2050	116.7		
2B	0.1261	72.5			0.1760	100.5		
2C	0.0841	48.9			0.1690	96.6		
2D	0.1013	57.5			0.1840	104.9		
2E	0.1255	71.9			0.1800	102.5		
2F	0.0783	44.6			0.1640	94.9		
3					0.0306	18.9	0.0320	19.8
4					0.0381	23.2	0.0357	21.9
5					0.2299	130.4	0.2182	124.1
6					0.0391	23.8	0.0320	19.8
7					0.1319	75.2	0.1249	71.9

(a) Homogenized in Waring Blender at 15,000 rpm

(b) Cell disruption by Parr Bomb

(c) Analyzed after thawing

(d) Optical density

(e) Dehydrogenase activity calculated from the empirical equation.

SECTION V

PRESENTATION AND DISCUSSION OF RESULTS

Batch Studies with E. coli:

A representative sample of the data obtained during the various batches utilizing glucose and galactose innoculated with E. coli are included in Table 5 through Table 15 and on Figure 9 through Figure 19. Each of these tables and figures indicate changes in substrate concentration, Coulter Counter enumeration, plate counts, suspended solids concentration and dehydrogenase activity as expressed by the empirical relationship or Equation 1 and Figure 8.

Various trends were established as the culture progressed through lag, log growth and eventually endogenous phase after the substrate was nearly depleted. Dehydrogenase activity was first observed at a limiting population of approximately 10^7 organisms/ml and increased during log growth. A similar behavior was observed for the solids analysis except, whereas the solids concentration reached a limiting value for any one batch and then leveled off for an extended period of time, dehydrogenase activity declined immediately after the substrate had been essentiallt depleted. The plate count and Coulter Counter data also followed dehydrogenase activity during log growth but plate counts decreased during the endogenous phase whereas the Coulter Counter enumeration remained more constant. Not until the batch studies had been extended for a considerable period beyond the time of depletion of the substrate did the counts and suspended solids concentrations begin to decrease. The refined analytical technique showed these trends more clearly in the later batches. (Appendix A)

The data in the tables and figures indicated that dehydrogenase activity as defined was a more sensitive indicator of the activity of the biomass with respect to its response to the growth limiting substrate during exponential growth as well as when the substrate had

reached a limiting concentration. During exponential growth, the plate count, Coulter Counter, suspended solids and dehydrogenase activity indicated similar trends. This similarity between parameters is illustrated on Table 26 which also suggested a change in ratio between suspended solids and dehydrogenase activity as the carbon source changed.

TABLE 5

Pure Culture Batch No. 1 With Glucose

Time, hour	Substrate, mg/l	Biomass Concentrations By			
		Dehydrogenase Activity, mg/l	Suspended Solids, mg/l	Plate Counts cells/ml	Coulter Counter cells, ml
0	230.0	-	-	1.2×10^6	1.3×10^7
1.75	154.0	38.0	130	1.5×10^7	1.5×10^7
2.16	127.0	-	-	-	-
2.50	124.0	54.0	140	1.1×10^7	2.1×10^7
2.75	113.0	61.0	180	1.8×10^7	2.2×10^7
67.00	-	21.0	-	1.3×10^7	4.3×10^7
117.0	-	18.3	-	-	-
157.00	-	18.7	220	2.0×10^3	7.1×10^6

TABLE 6

Pure Culture Batch No. 2 With Glucose

Time, hour	Substrate, mg/l	Biomass Concentrations By			
		Dehydrogenase Activity, mg/l	Suspended Solids, mg/l	Plate Counts Cells/ml	Coulter Counter cells/ml
0	420.0	-	-	1.2×10^6	1.9×10^7
1.75	324.0	38.0	160	1.5×10^7	2.2×10^7
2.16	-	85.0	180	1.6×10^7	2.4×10^7
2.50	222.0	123.0	190	1.6×10^7	2.1×10^7
2.75	214.0	121.0	-	2.3×10^7	3.0×10^7
67.00	-	31.0	-	1.28×10^7	-
117.00	-	21.6	-	-	-
157.00	-	21.0	220	1.7×10^5	1.03×10^7

TABLE 7

Pure Culture Batch No. 3 With Glucose

Time, hour	Substrate, mg/l	Biomass Concentrations By			
		Dehydrogenase Activity, mg/l	Suspended Solids, mg/l	Plate Counts cells/ml	Coulter Counter cells/ml
0	1,475.0	-	-	1.42×10^7	1.9×10^6
3.00	1,125.0	18.9	-	1.20×10^7	2.0×10^6
5.00	1,150.0	23.2	30	8.0×10^6	2.3×10^6
6.00	1,092.0	52.7	55	2.0×10^7	7.8×10^6
7.00	948.0	130.4	125	4.0×10^7	4.4×10^7
7.50	782.0	-	-	-	-
12.50	312.0	156.0	-	-	-
21.00	253.0	-	-	-	-
21.75	188.0	-	-	-	-
23.25	145.0	65.1	230	2.2×10^8	1.2×10^7
24.50	143.0	60.5	180	2.4×10^8	1.1×10^7
26.25	110.5	52.2	220	1.8×10^8	8.4×10^6
29.00	64.4	-	140	4.0×10^8	1.4×10^7
30.00	33.5	25.4	-	6.6×10^7	5.2×10^6
49.50	-	-	85	3.0×10^7	6.4×10^6
75.00	-	-	80	3.0×10^7	4.3×10^6
107.00	-	-	190	3.4×10^5	8.7×10^6

TABLE 8

Pure Culture Batch No. 4 With Galactose

Time, hour	Substrate, mg/l	Biomass Concentrations By			
		Dehydrogenase Activity, mg/l	Suspended Solids, mg/l	Plate Counts cells/ml	Coulter Counter cells/ml
0	583.0	-	165	1.76×10^8	3.4×10^6
1.50	335.0	-	-	2.18×10^8	3.2×10^6
2.50	-	36.0	180	2.71×10^8	3.8×10^6
5.00	238.0	46.0	210	2.64×10^8	5.7×10^6
7.00	231.0	69.3	-	2.83×10^8	6.9×10^6
14.50	116.0	-	-	-	-
30.50	-	48.9	200	3.60×10^8	9.4×10^6
55.00	-	42.0	170	-	8.2×10^6
76.00	-	37.7	280	7.4×10^8	1.7×10^7
100.50	-	24.6	230	1.5×10^8	2.9×10^6
146.25	-	-	270	-	3.8×10^7

TABLE 9

Pure Culture Batch No. 5 With Galactose

Time, hour	Substrate, mg/l	Biomass Concentrations By			
		Dehydrogenase Activity, mg/l	Suspended Solids, mg/l	Plate Counts cells/ml	Coulter Counter cells/ml
0.25	924.0	86.7	320	5.0×10^7	7.4×10^6
3.25	752.0	150.3	270	3.3×10^8	1.0×10^7
5.00	731.0	101.9	340	5.2×10^8	1.23×10^7
6.50	565.0	109.0	-	6.9×10^8	1.20×10^7
12.50	94.8	86.3	298	-	-
31.00	52.7	53.0	-	2.1×10^9	1.60×10^8
52.50	-	69.1	390	6.9×10^8	3.40×10^7
77.50	-	41.1	557	5.3×10^8	3.2×10^7
132.25	-	-	530	2.3×10^5	-

TABLE 10

Pure Culture Batch No. 6 With Galactose

Time, hour	Substrate, mg/l	Biomass Concentrations By			
		Dehydrogenase Activity, mg/l	Suspended Solids, mg/l	Plate Counts cells/ml	Coulter Counter cells/ml
0.25	1,240.0	231.9	430	5.7×10^8	2.6×10^7
3.25	803.0	233.4	490	9.2×10^8	3.2×10^7
5.00	348.0	121.1	530	6.0×10^8	2.6×10^7
6.50	93.2	125.3	500	1.0×10^9	4.1×10^7
12.50	42.6	75.2	567	-	-
31.00	-	113.4	510	1.7×10^9	6.7×10^7
52.50	-	117.7	580	5.5×10^8	7.4×10^7
77.50	-	105.4	600	7.1×10^8	9.2×10^7
126.00	-	24.0	430	5.6×10^5	-

TABLE 11

Pure Culture Batch No. 7 With Sucrose

Time, hour	Sucrose,* mg/l	Biomass Concentrations By			
		Dehydrogenase Activity, mg/l	Suspended Solids, mg/l	Plate Counts cells/ml	Coulter Counter cells/ml
0	2,170	-	-	1.5×10^6	4.6×10^7
1.50	2,170	12.1	-	3.8×10^6	1.1×10^7
2.50	2,210	19.6	-	7.5×10^6	3.4×10^7
3.50	1,990	26.1	-	4.0×10^7	-
4.50	1,935	131.4	150	4.6×10^7	3.5×10^9
6.00	1,692	91.6	130	9.1×10^8	6.1×10^9
7.00	1,620	61.6	130	9.3×10^8	7.1×10^9
25.00	1,325	43.5	-	7.5×10^9	1.5×10^{10}
29.00	-	-	400	9.4×10^0	1.7×10^{10}
35.50	884	41.5	-	-	-
47.00	847	52.6	660	-	2.0×10^{10}

* measured by Chemical Oxygen Demand (Standard Methods)

TABLE 12

Pure Culture Batch No. 8 With Acetic Acid

Time, hour	Substrate, mg/l		Biomass Concentrations			
	Acetic Acid	Total Organic Carbon	Dehydrogenase Activity, mg/l	Suspended Solids, mg/l	Plate Counts cell/ml	Coulter Counter cell/ml
0	1,254	553	84.1	120	4.2×10^8	2.6×10^8
0.50	-	-	77.4	130	6.9×10^8	4.4×10^8
1.50	1,134	517.4	-	170	7.6×10^8	8.4×10^8
3.00	-	-	-	-	9.7×10^8	1.2×10^9
4.25	-	-	159.9	190		
6.00	826	430	165.5	340	9.6×10^8	1.7×10^9
7.00	774	384.6	224.4	350	1.0×10^8	2.3×10^9
24.50	-	-	[illegible].9	320		
29.00	60	103.8	-	-	9.8×10^8	4.7×10^9

TABLE 13

Pure Culture Batch No. 9 With Acetic Acid

Time, hour	Acetic Acid, mg/l	Biomass Concentrations			
		Dehydrogenase Activity, mg/l	Suspended Solids, mg/l	Plate Counts cell/ml	Coulter Counter cell/ml
0	2,250	-	33.3	6.7×10^7	4.9×10^7
2	2,100	11.0	53.2	1.1×10^8	8.1×10^7
3	2,222	14.9	59.8	1.3×10^8	9.5×10^7
5	2,100	21.0	54.4	1.2×10^8	1.8×10^8
6	2,100	38.3	90.0	3.4×10^8	4.6×10^8
7.5	2,100	60.6	190.0	6.2×10^8	7.1×10^8
14.75	-	238.6	330.0	-	-
24.0	1,080	507.6	380.0	1.5×10^9	6.2×10^9
26.0	1,020	446.0	470.0	2.6×10^9	5.4×10^9
29.0	640	527.0	500.0	2.2×10^9	4.6×10^9
48.0	380	856.0	720.0	1.5×10^9	5.3×10^9
55.0	-	514.0	-	-	-
79.0	-	28.6	560.0	-	-

TABLE 14

Pure Culture Batch No. 10 With L-Alanine

Time, hour	L-Alanine		Biomass Concentrations			
	Total Organic Carbon, mg/l	Organic Nitrogen, mg/l	Dehydrogenase Activity, mg/l	Suspended Solids, mg/l	Plate Count cell/ml	Coulter Count cell/ml
0	441.6	82	177.6	230	6.7×10^8	6.8×10^8
1.0	420	76	225.4	340	7.8×10^8	8.4×10^8
2.0	378	70	179.0	360	1.3×10^9	1.1×10^9
4.50	330	51	172.8	290	2.1×10^9	1.8×10^9
6.00	288	-	260.9	370	3.9×10^9	1.8×10^9
24.50	129.6	-	149.5	390	2.2×10^9	3.5×10^9
29.50	100.5	-	-	-	3.5×10^9	

TABLE 15

Pure Culture Batch No. 11 With Benzoic Acid

Time, Hour	Dehydrogenase, Activity, mg/l	Suspended Solids, mg/l	Plate Counts Cell/ml	Coulter Counter counts/ml
0	81.4	280	4.9×10^8	1.2×10^9
0.75	70.8	260	5.8×10^8	7.9×10^8
2.50	138.6	320	3.0×10^8	8.4×10^8
3.25	151.9	400	8.0×10^8	8.8×10^8
4.50	130.7	320	1.2×10^9	9.3×10^8
6.00	100.6	360	6×10^8	9.7×10^8
11.00	125.6	580	---	---
29.00	154.4	---	2.5×10^9	---
52.00	182.0	620	1.3×10^9	---
75.75	111.4	620	6.7×10^8	---
95.75	98.1	600	1.6×10^8	---
166.00	7.8	---	---	---

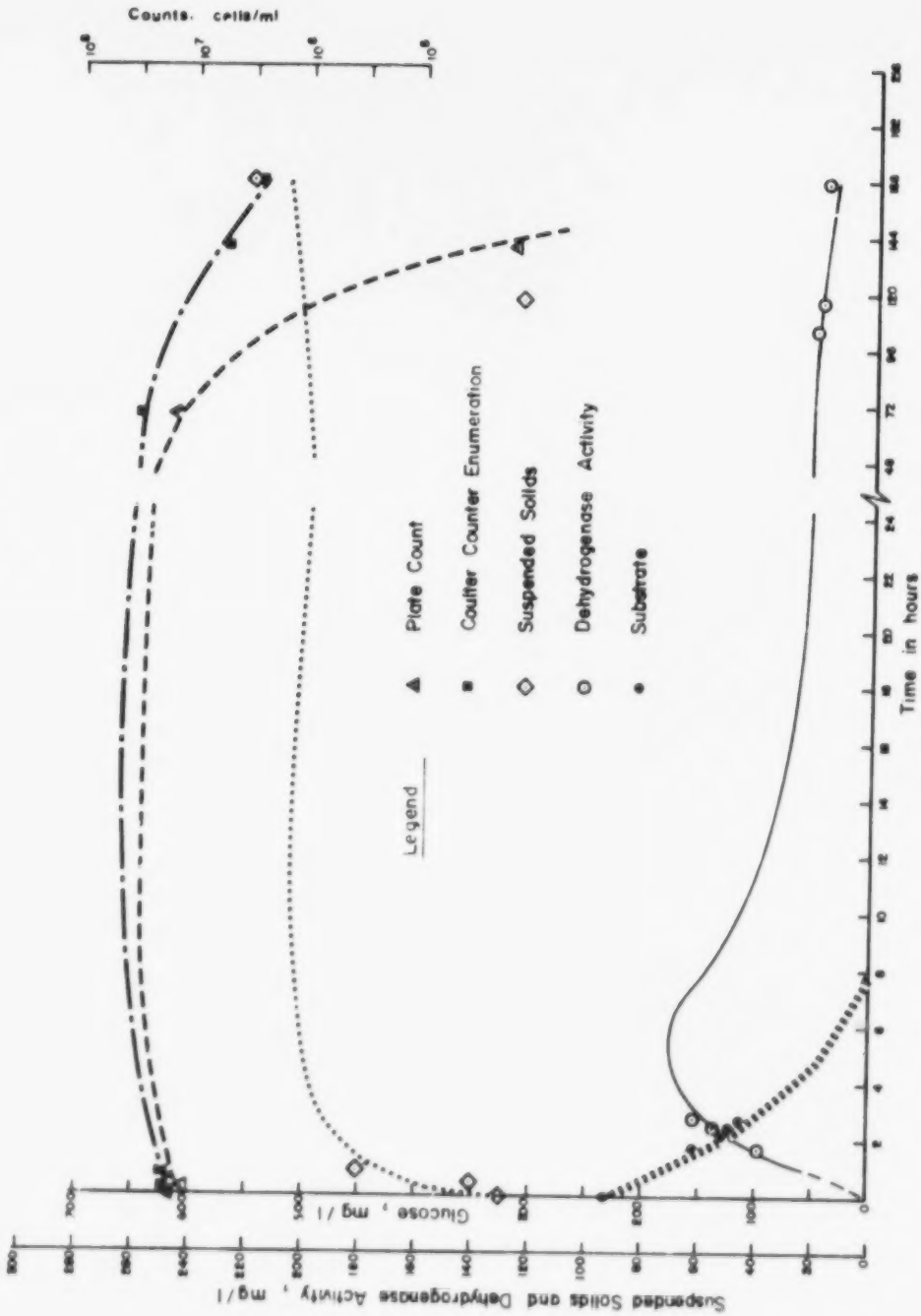

Figure 9. Pure Culture Batch No. 1 With E. Coli and Glucose Substrate

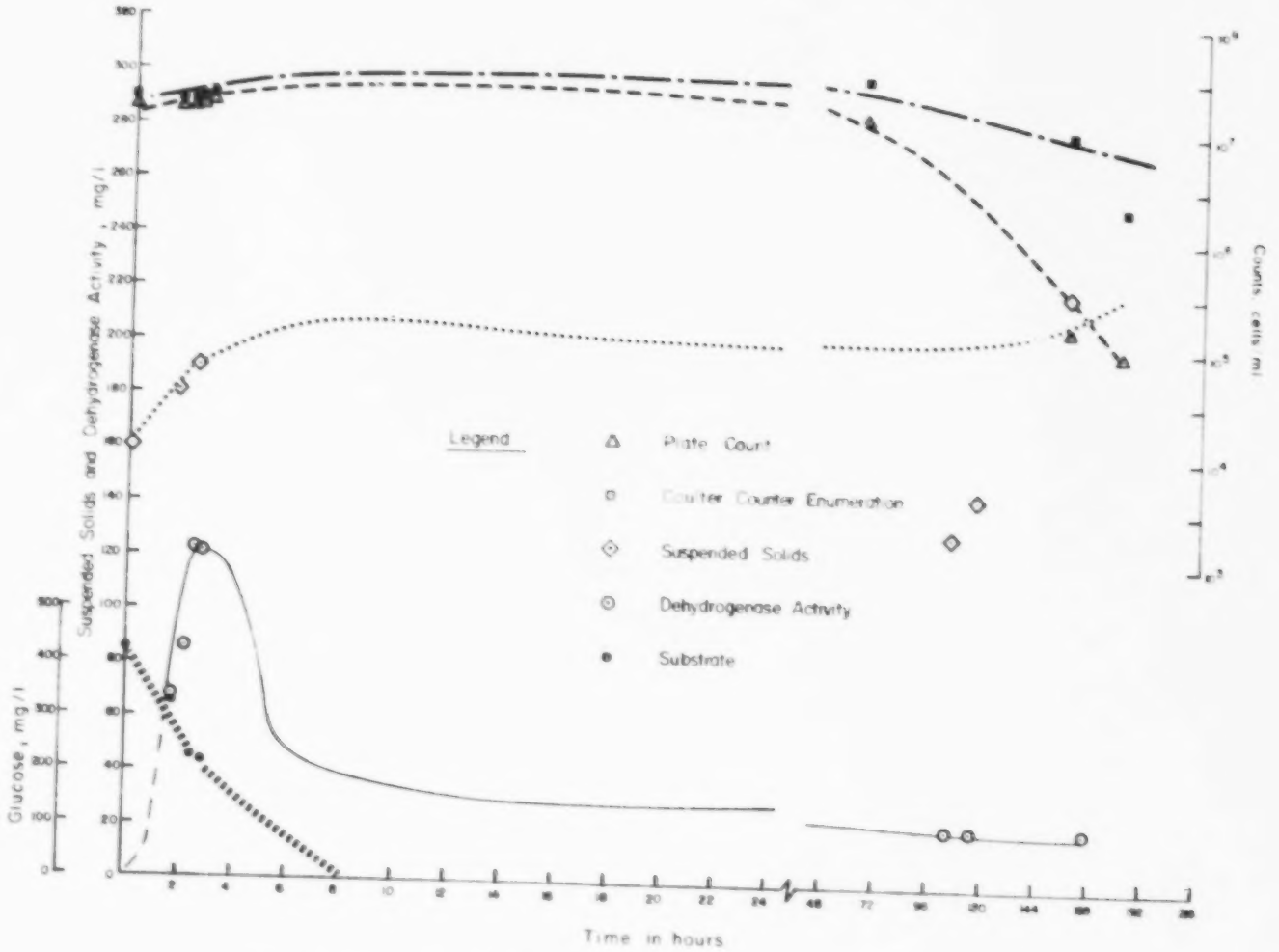

Figure 10. Pure Culture Batch No. 2 With E. Coli and Glucose Substrate

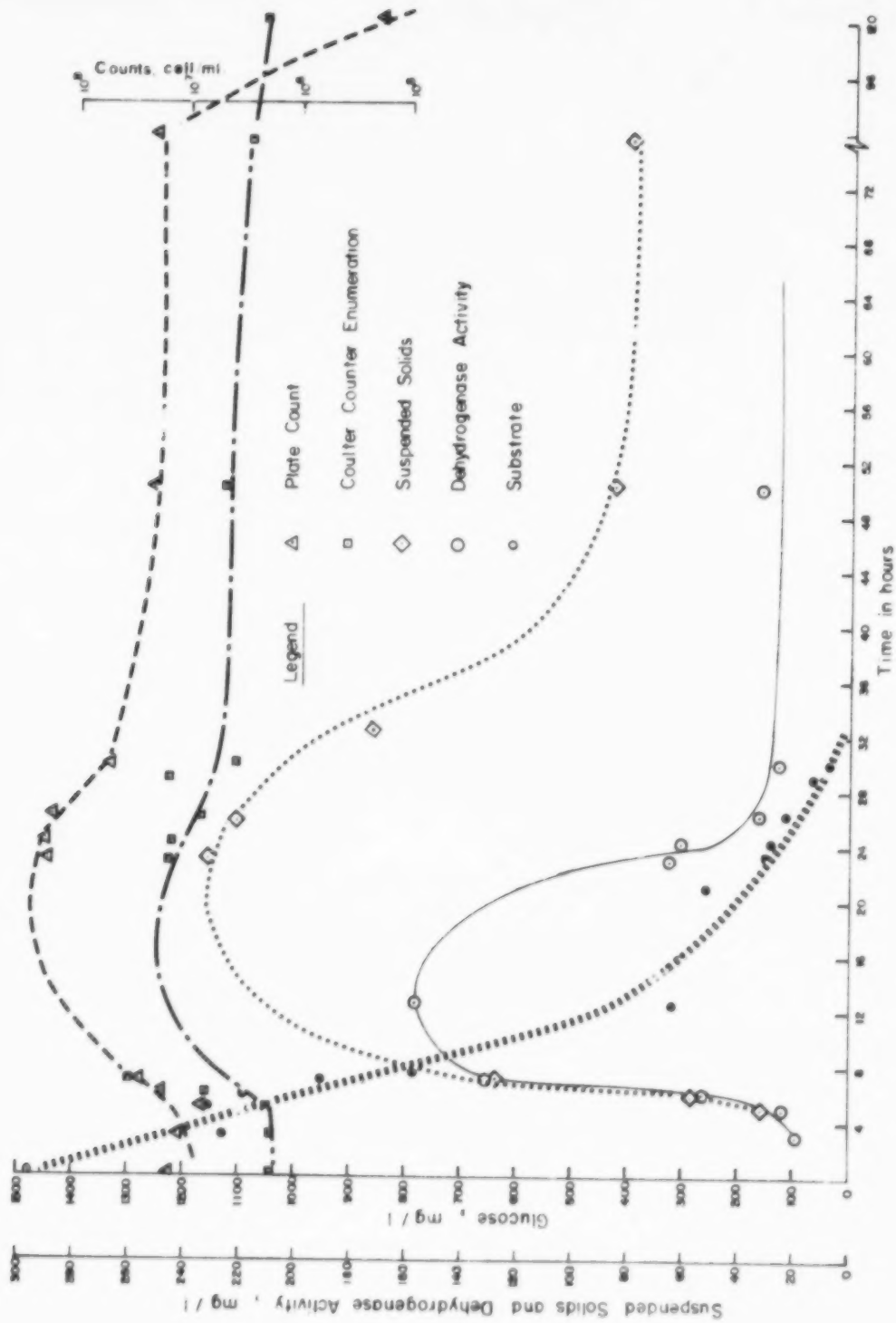

Figure 11. Pure Culture Batch No. 3 With E. Coli and Glucose Substrate

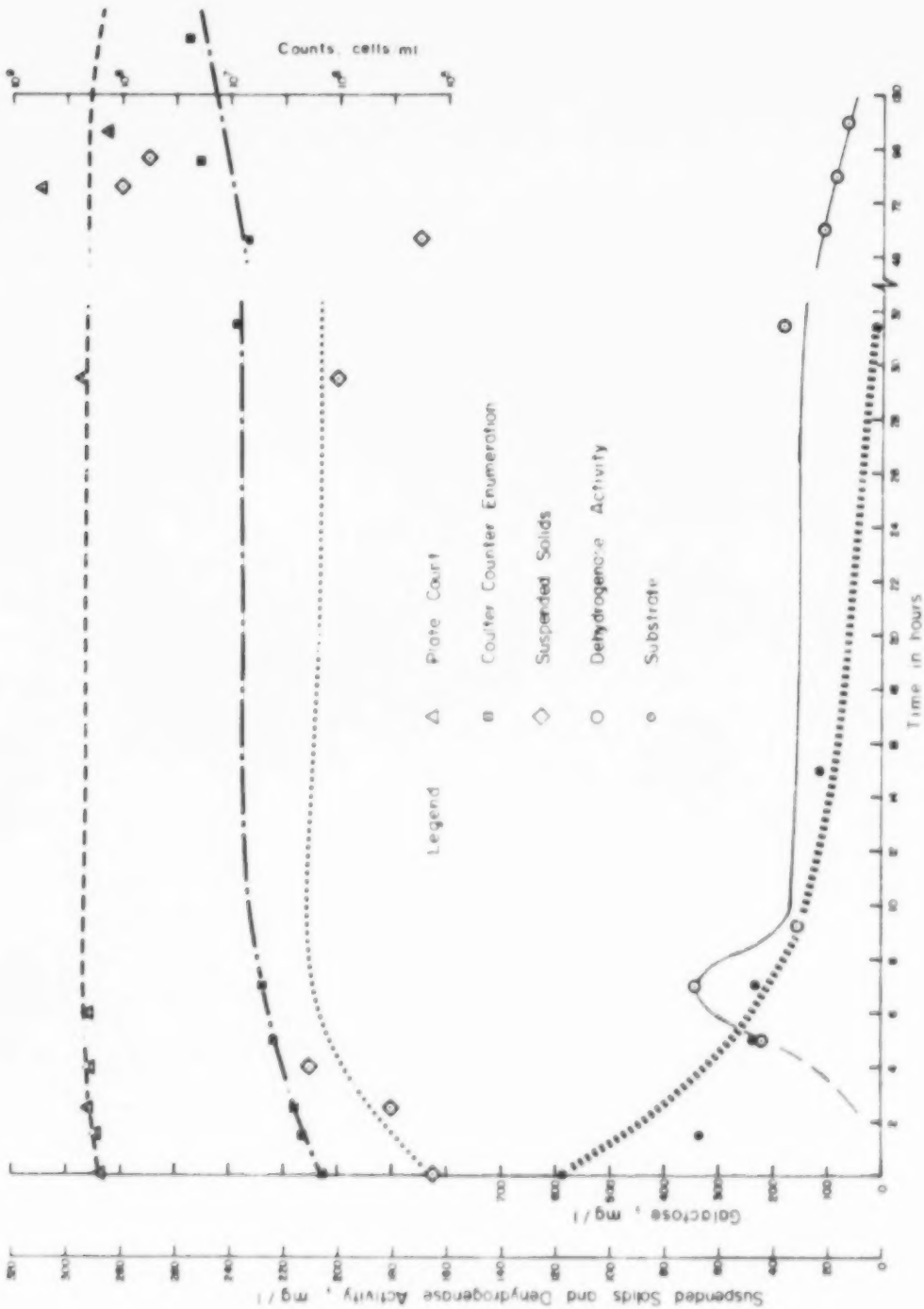

Figure 12. Pure Culture Batch No. 4 With E. Coli and Galactose Substrate

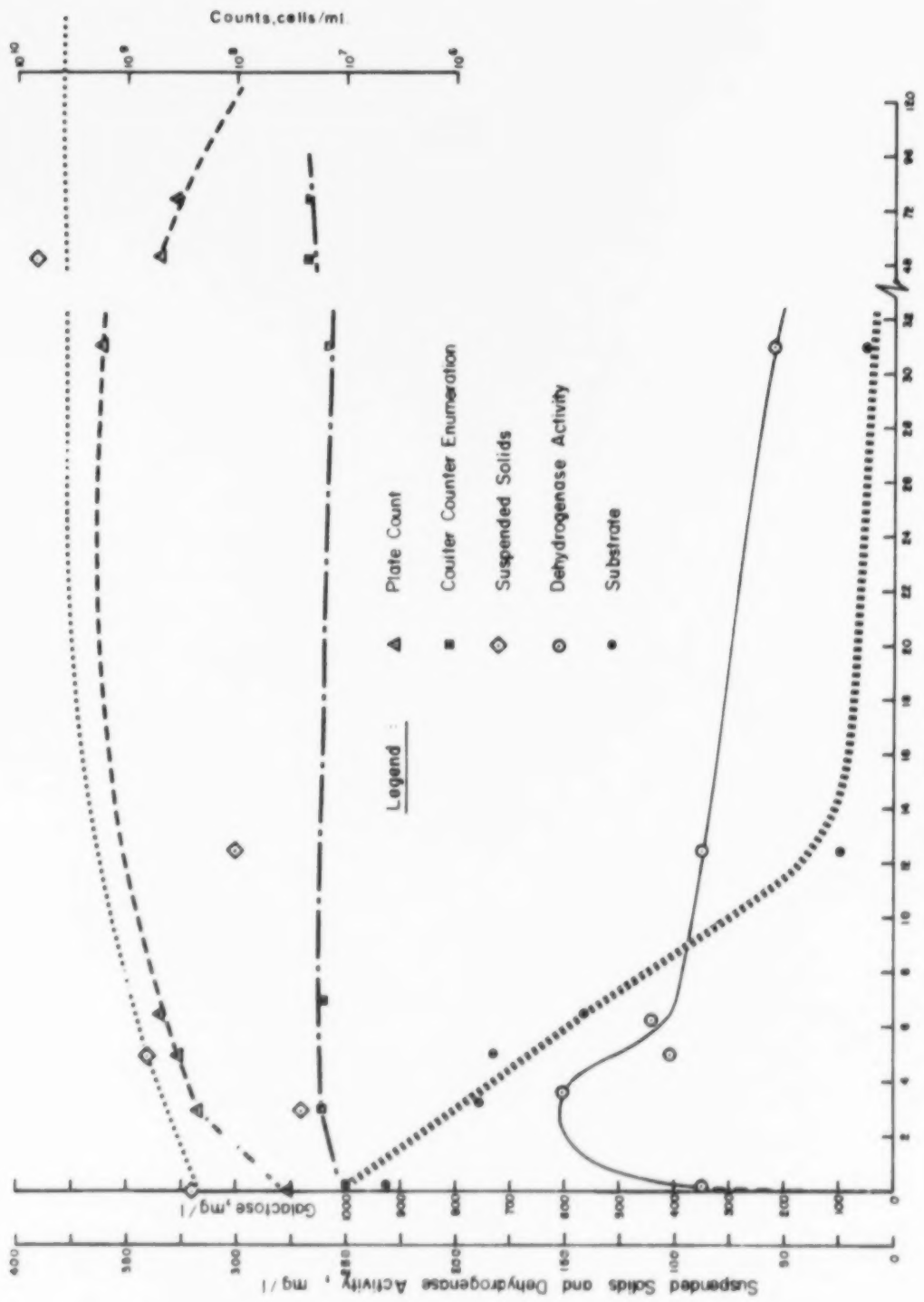

Figure 13. Pure Culture Batch No. 5 With E. Coli and Galactose Substrate

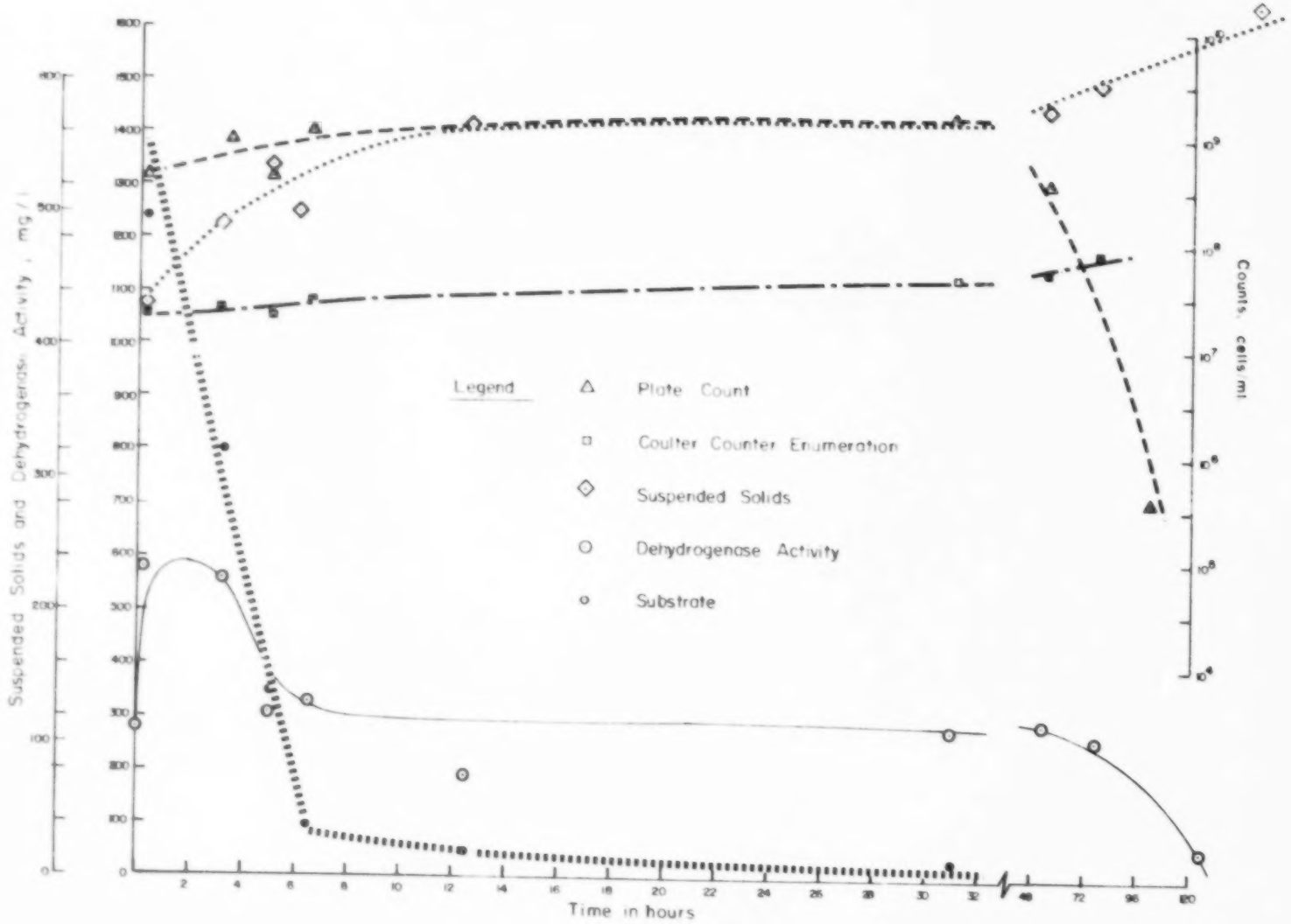

Figure 14. Pure Culture Batch No. 6 With E. Coli and Galactose Substrate

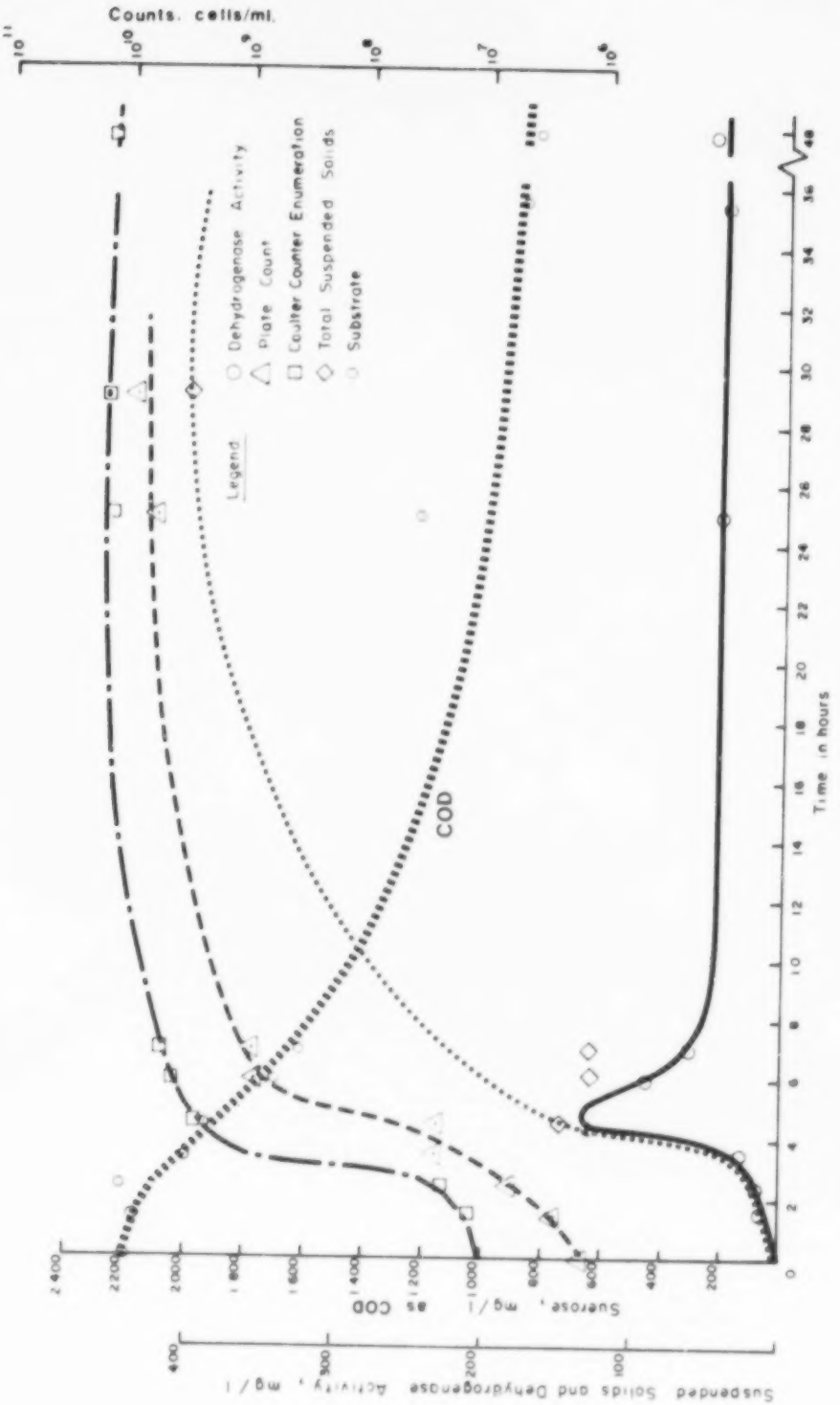

Figure 15. Pure Culture Batch No. 7 With E. Coli and Sucrose Substrate

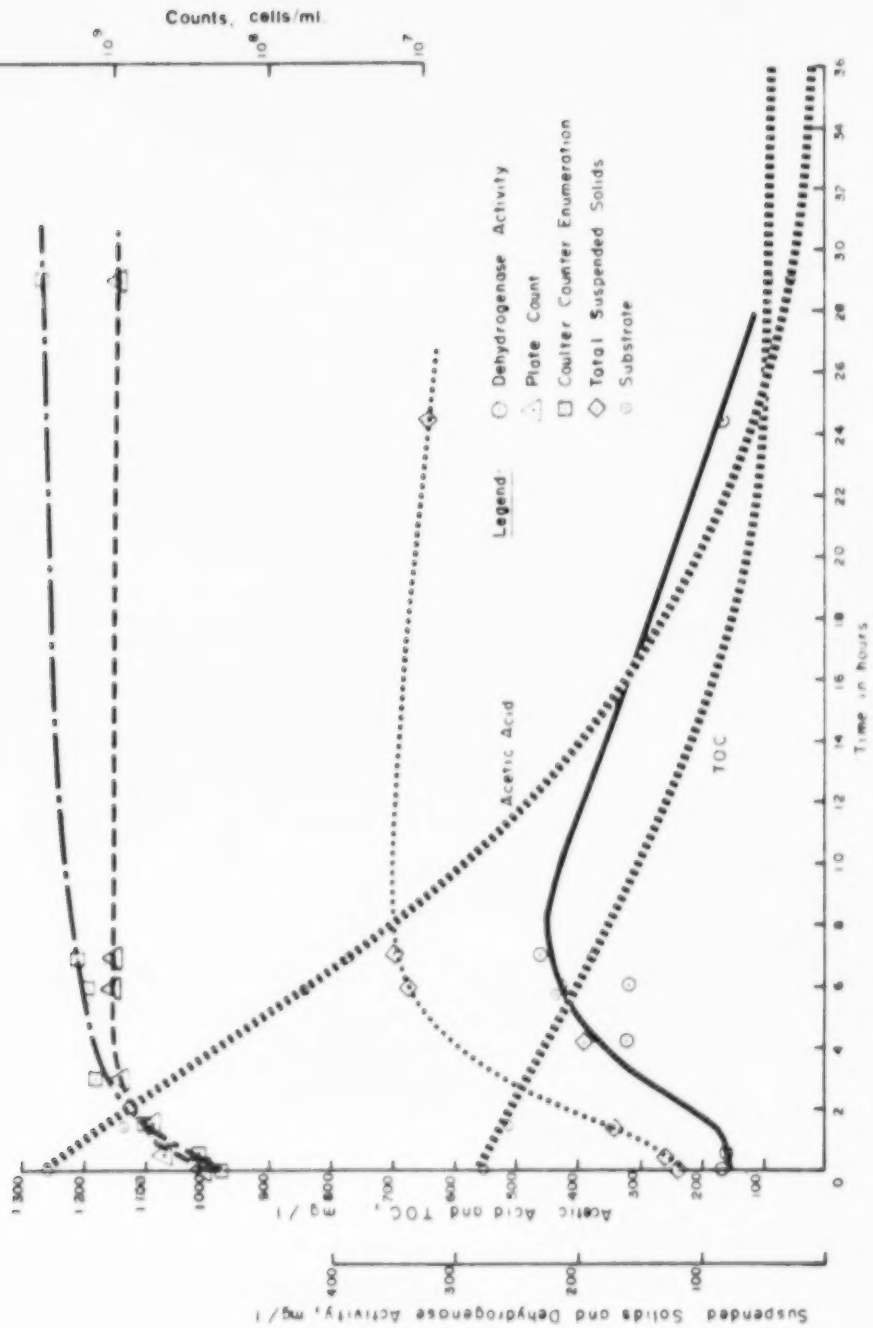

Figure 16. Pure Culture Batch No. 8 With E. Coli and Acetic Acid Substrate

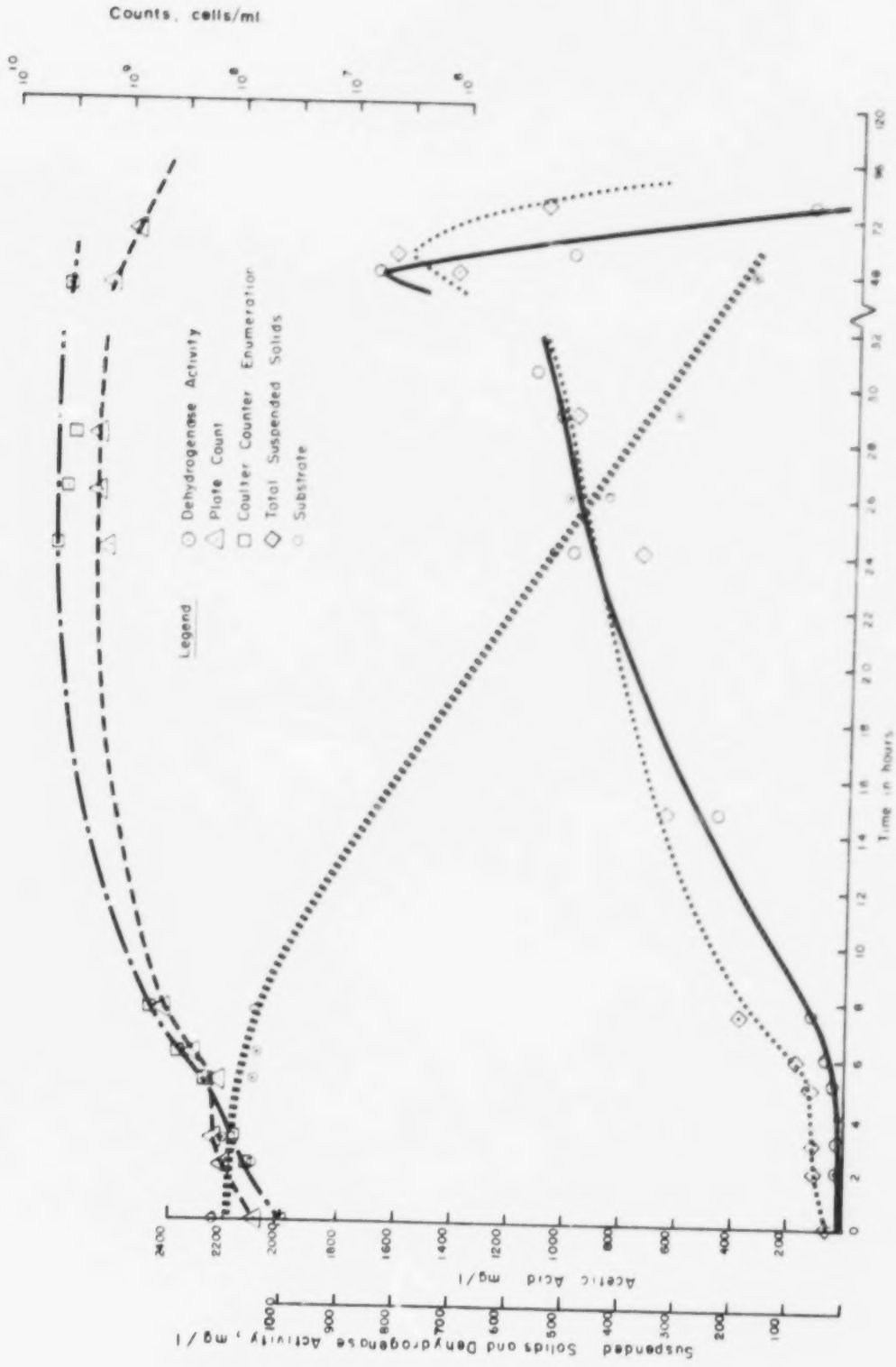

Figure 17. Pure Culture No. 9 With E. Coli and Acetic Acid Substrate

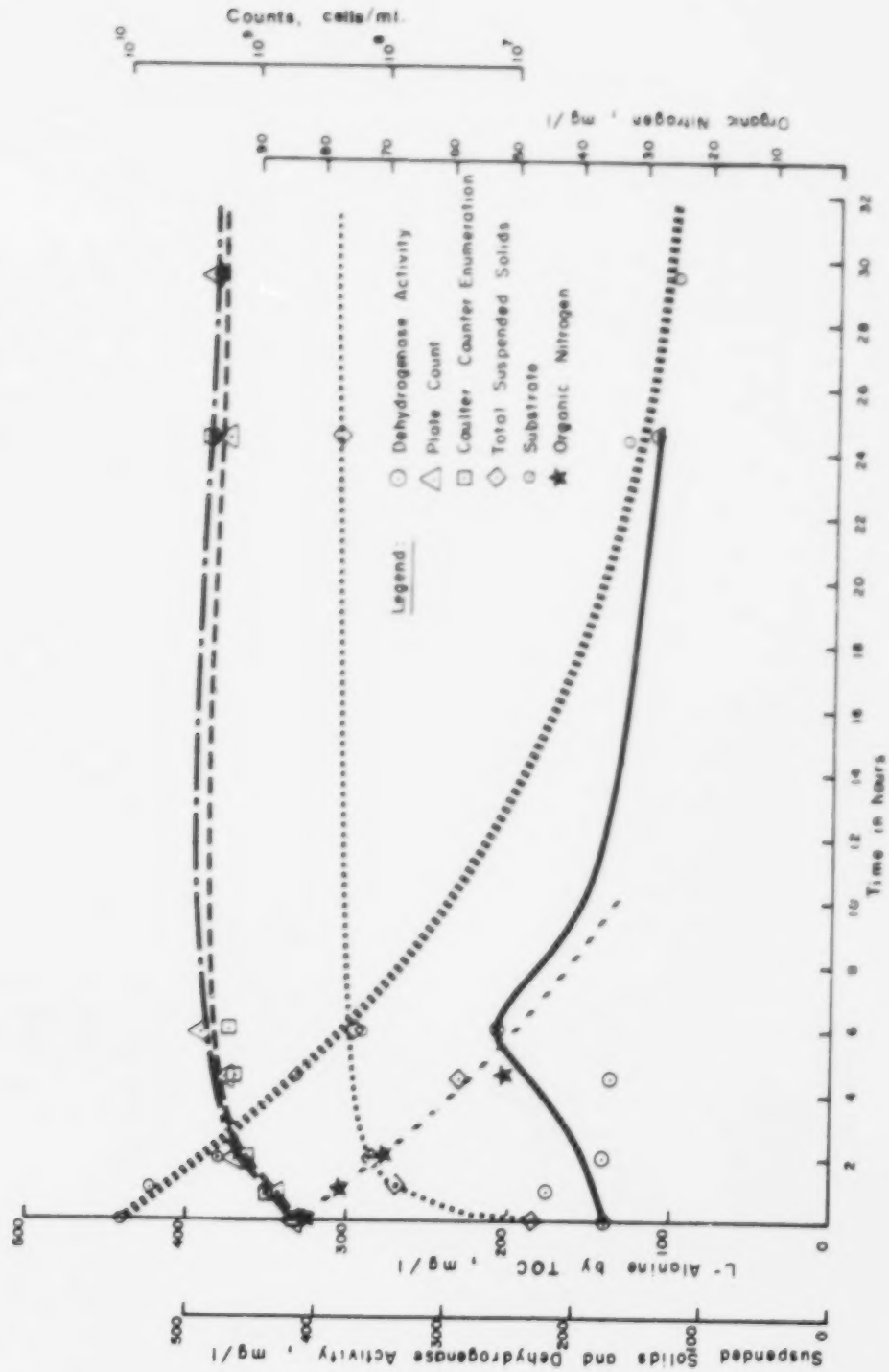

Figure 18. Pure Culture Batch No. 10 With E. Coli and L-Alanine Substrate

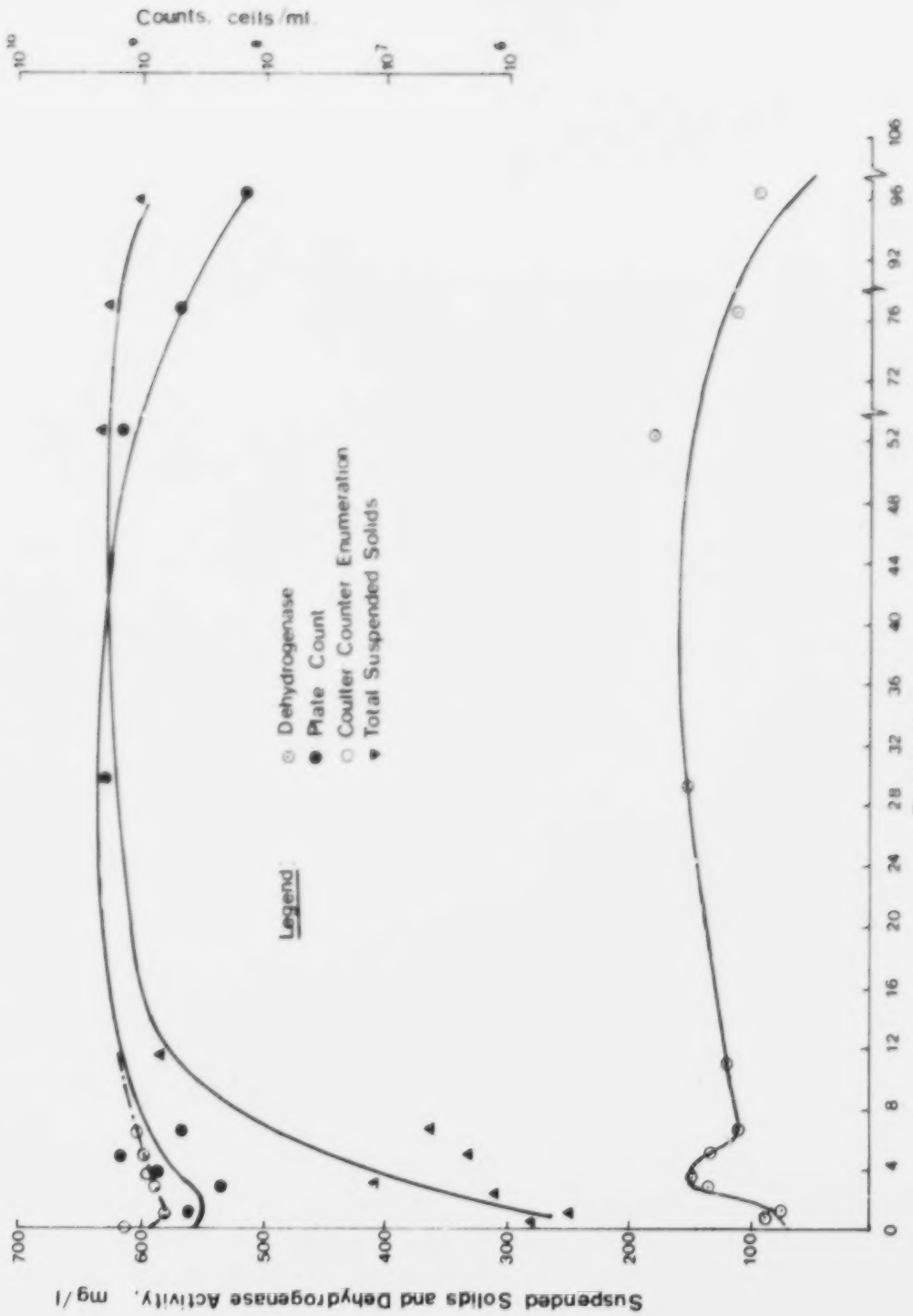

Figure 19. Pure Culture Batch No. 11 With E. Coli and Benzoic Acid Substrate

Batch studies with Heterogeneous Cultures:

Seven batch studies with heterogeneous aerobic cultures were conducted with glucose, galactose, sucrose, acetic acid, and L-alanine substrates. The data in Table 16-22 and Figures 20-26 indicated that dehydrogenase activity was a very sensitive indicator of the activity of the biomass. With the addition of ATP analysis, Figures 25 and 26 indicated the same pattern between dehydrogenase activity and ATP concentrations throughout the growth phase. A comparison at ATP content extracted by dimethyl sulfoxide (DMSO) solvent and nitrogen bombing was also included in Figures 25 and 26. A parallel relationship between the extraction technique was reflected by curves. During exponential growth, suspended solids and dehydrogenase activity maintained a similar trend as observed in the pure culture studies as shown in Table 26.

In addition to the aerobic studies, an anaerobic digester was maintained to demonstrate the possible applicability of the dehydrogenase test in such a process. The digester had been fed with leachate from a solid waste disposal site once a day and with initial COD concentrations of 350,550, and 800 mg/l.

The data from three batch studies are included in Figures 27,28 and 29 and Tables 23,24, and 25. Leachate concentrations, biomass by all parameters and gas (carbon dioxide and methane) production were measured. It was during these batch studies that the sensitivity of the dehydrogenase test became more pronounced when compared with other parameters. The total suspended solids with both non-volatile and volatile fractions including biomass did not reflect changes interpretable in terms of activities of organisms in the system. The magnitude of increase in solids concentration after 8-10 hours was much less than observed in aerobic systems.

ATP concentrations were monitored in two of these batch studies and showed a rapid decrease instead of an increase in the first six hours followed by a slight increase (Figures 24 and 25). Similar observations on ATP were reported by Forrest[39] during the growth of the anaerobic,

Streptococcus faecalis, on a pyruvate substrate. Apparently, during the first several hours of the experiment after growth began, synthesis reactions made heavy demands on the ATP pool causing a rapid decrease in the pool level. It appeared then to fall below the critical level necessary to sustain exponential growth. Consequently linear growth took place, limited by the availability of ATP for synthesis.

Contrary to these observations on ATP content or solids concentrations, the dehydrogenase activity in the studies reported herein was consistently sensitive to the behavior of the active biomass during the growth cycle under anaerobic conditions. As the substrate was depleted and the corresponding gas production rate decreased, the dehydrogenase activity also decreased to a minimum value.

TABLE 16

Heterogeneous Culture Batch No. 1 With Glucose

Time, hour	Glucose, mg/l	Biomass Concentrations		
		Dehydrogenase Activity, mg/l	Total Suspended Solids, mg/l	Volatile Suspended Solids, mg/l
0	1540	19	40	40
1.50	1252	24.8	65	60
2.75	948	33.2	90	75
5.50	153	47.0	120	110
6.25	70.2	60.0	-	-
7.75	8.1	4.14	150	110
24.00	0	20.1	185	135
31.00	0	16.2	225	145

TABLE 17

Heterogeneous Culture Batch No. 2 With Glucose

Time, hour	Glucose, mg/l	Biomass Concentrations		
		Dehydrogenase Activity, mg/l	Total Suspended Solids, mg/l	Volatile Suspended Solids, mg/l
0	824	47.2	65	50
1.25	739	89.3	120	90
2.75	671	134.4	190	140
4.75	563	95.1	215	160
6.25	429	96.3	220	130
13.25	189	97.4	250	175
23.75	7.2	101.8	-	-
28.50	0	102.3	-	-
30.00	0	74.3	250	170
53.50	0	74.3	220	-
72.00	0	31.8	195	180
79.00	0	20.4	160	145

TABLE 18

Heterogeneous Culture Batch No. 3 With Galactose

Time, hour	Galactose, mg/l	Biomass Concentrations		
		Dehydrogenase Activity, mg/l	Total Suspended Solids, mg/l	Volatile Suspended Solids, mg/l
0	740	-	-	-
1.0	737	-	30	25
2.50	751	-	25	15
4.50	742	19.0	130	80
6.25	691	33.0	145	100
7.25	534	42.4	230	190
8.50	162	91.0	250	190
11.50	24.8	116.2	280	190
13.50	12.2	166.4	290	225
24.00	7.3	85.9	250	230
26.75	-	69.8	330	300
29.50	-	70.4	190	180
32.50	-	-	230	210
48.00	-	15.7	110	90
52.25	-	12.0	110	90

TABLE 19

Heterogeneous Culture Batch No. 4 With Galactose

Time, hour	Galactose, mg/l	Biomass Concentrations		
		Dehydrogenase Activity, mg/l	Total Suspended Solids, mg/l	Volatile Suspended Solids, mg/l
0	942	-	-	-
1.25	928	-	-	-
2.75	1010	-	15	5
4.75	950	11.6	-	-
6.00	842	15.2	15	-
7.00	-	32.1	-	-
8.50	788	-	85	50
12.25	763	130.6	140	140
14.50	595	184.4	155	185
23.50	161	148.2	320	280
26.00	-	131.6	-	-
29.50	6.4	88.8	320	305
30.50	-	-	315	285
48.50	-	72.6	285	260
53.50	-	-	265	-
73.00	-	20.8	-	170
77.00	-	-	-	155

TABLE 20

Heterogeneous Culture Batch No. 5 With Sucrose

Time, hour	Sucrose by COD, mg/l	Biomass Concentrations By		
		Dehydrogenase Activity, mg/l	Total Suspended Solids, mg/l	Volatile Suspended Solids, mg/l
0	2440	19.3	120	120
1.0	2400	21.0	115	100
2.0	2360	23.5	135	125
3.5	2360	28.1	160	135
5.0	--	27.7	170	165
7.0	2280	112.6	205	175
26.25	2020	14.4	175	170
29.5	1940	15.8	230	200

TABLE 21

Heterogeneous Culture Batch No. 6 With Acetic Acid

Time, hour	Acetic Acid by Total Organic Carbon, mg/l	Dehydrogenase Activity, mg/l	Biomass Concentration By			
			Total Suspended Solids, mg/l	Volatile Suspended Solids, mg/l	ATP, fg/ml	
					DMSO	Bombing
0	940	-	-	-	1.55×10^7	2.02×10^6
1.75	-	-	250	100	1.93×10^7	2.77×10^6
4.50	-	18.0	188.5	115	1.36×10^7	3.00×10^6
6.25	325	-	192.5	140	1.51×10^7	1.39×10^6
7.50	308	24.8	225	190	1.92×10^7	3.00×10^6
24.00	30	65.7	225	205	2.32×10^8	7.17×10^7
25.75	19	34.9	250	210	1.36×10^8	3.63×10^7
27.75	18	26.1	300	205	8.86×10^7	2.96×10^7
48.00	-	0	265	145	2.89×10^7	4.61×10^6

TABLE 22

Heterogeneous Culture Batch No. 7 With L-Alanine

Time, hour	L-Alanine by Total Organic Carbon, mg/l	Biomass Concentrations By				
		Dehydrogenase Activity, mg/l	Total Suspended Solids, mg/l	Volatile Suspended Solids, mg/l	ATP, fg/ml, by	
					DMSO	Bombing
0	420	0	-	-	-	-
1.0	396	9.8	100	60	2.00×10^8	1.26×10^7
3.0	-	20.8	90	-	2.12×10^8	1.63×10^7
4.5	288	66.3	-	-	1.56×10^8	2.07×10^7
6.0	184	87.3	165	140	2.12×10^8	3.79×10^7
7.5	126	122.6	180	130	2.83×10^8	3.88×10^7
9.0	-	130.0	245	265	3.35×10^8	5.6×10^7
15.0	56	113.6	510	405	6.06×10^8	9.92×10^7
25.0	-	47.2	500	170	-	2.10×10^7
29	27.5	19.3	215	110	2.09×10^8	3.83×10^7

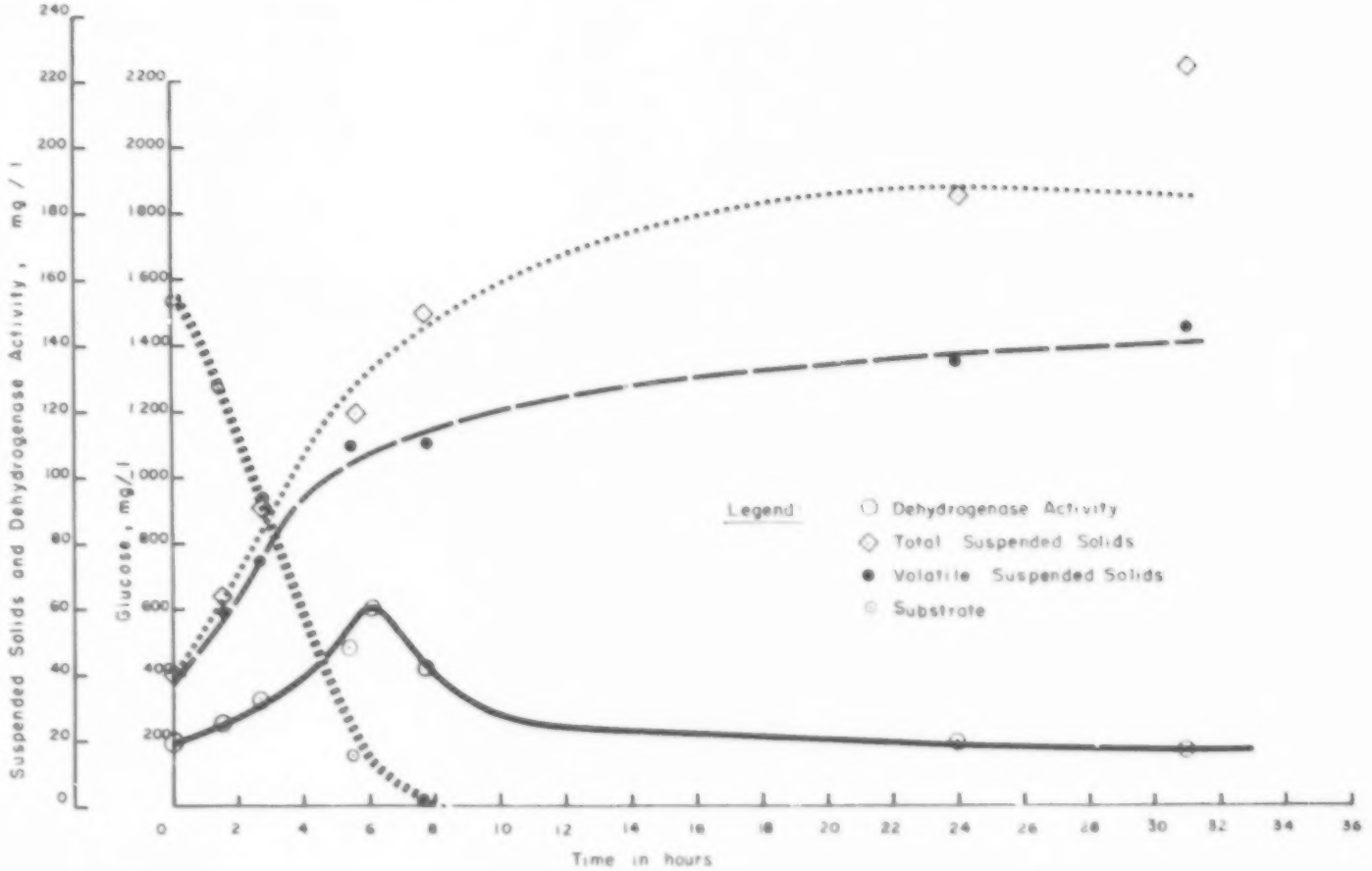

Figure 20. Heterogenous Culture Batch No. 1 With Glucose Substrate

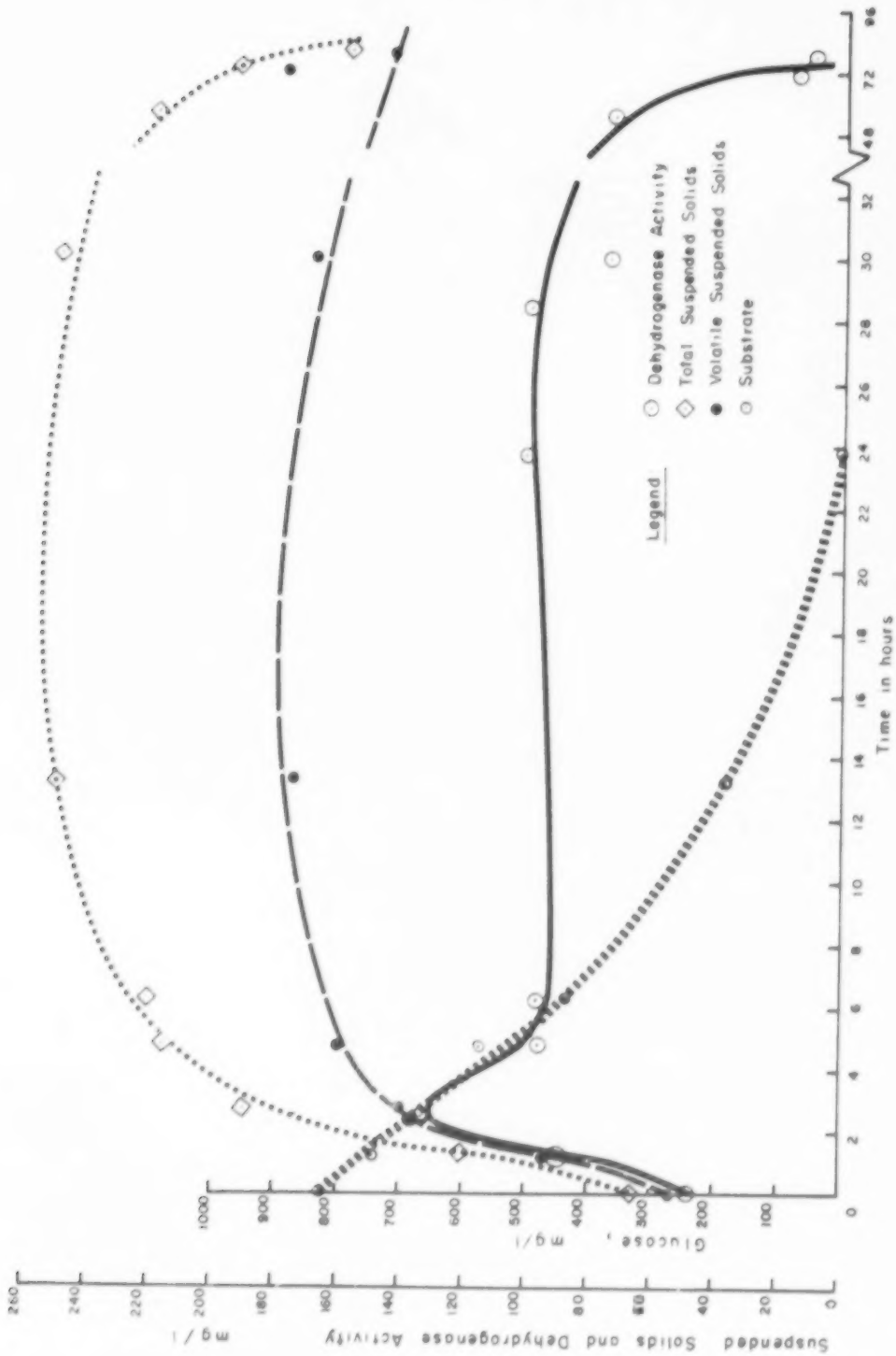

Figure 21. Heterogeneous Culture Batch No. 2 With Glucose Substrate

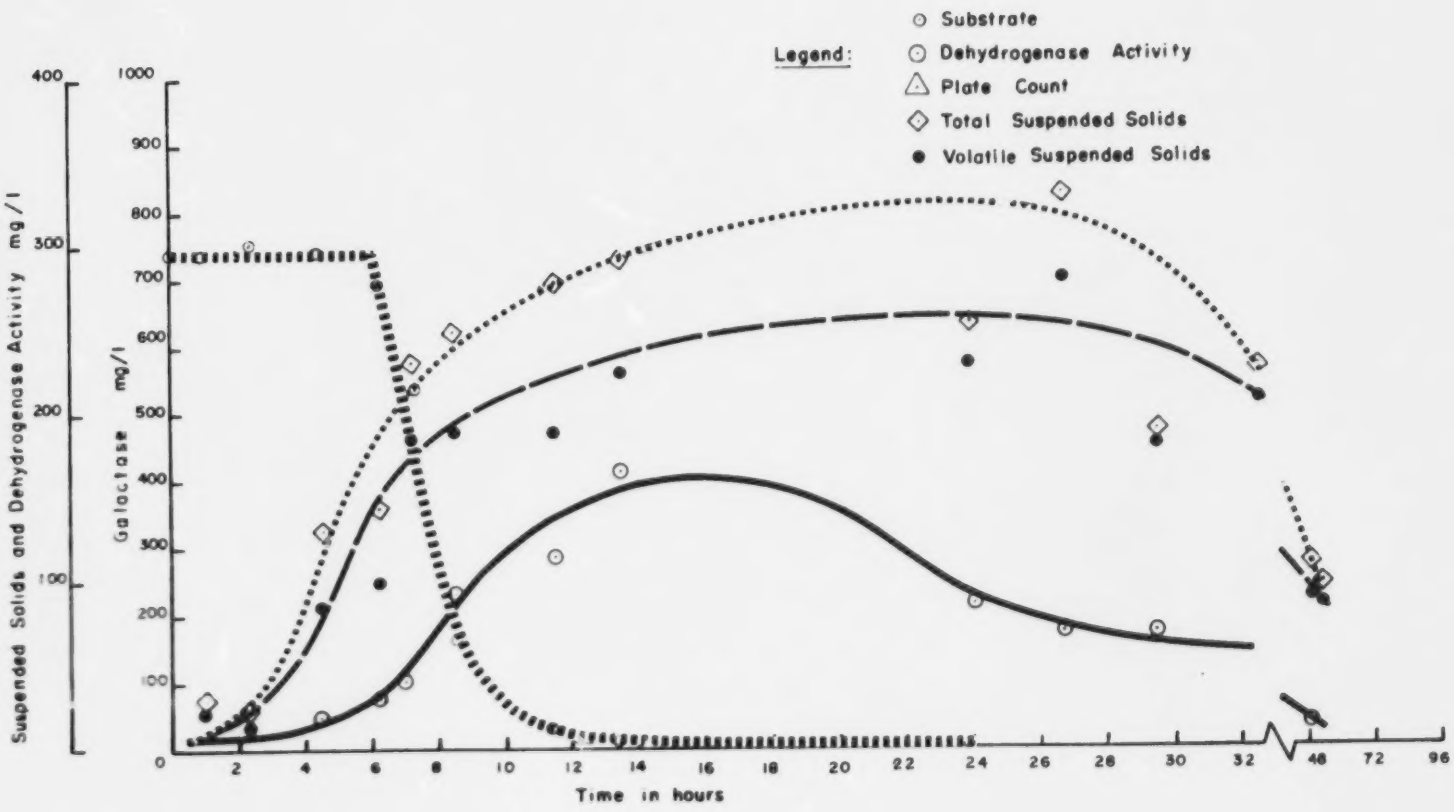

Figure 22. Heterogeneous Culture Batch No. 3 Galactose Substrate

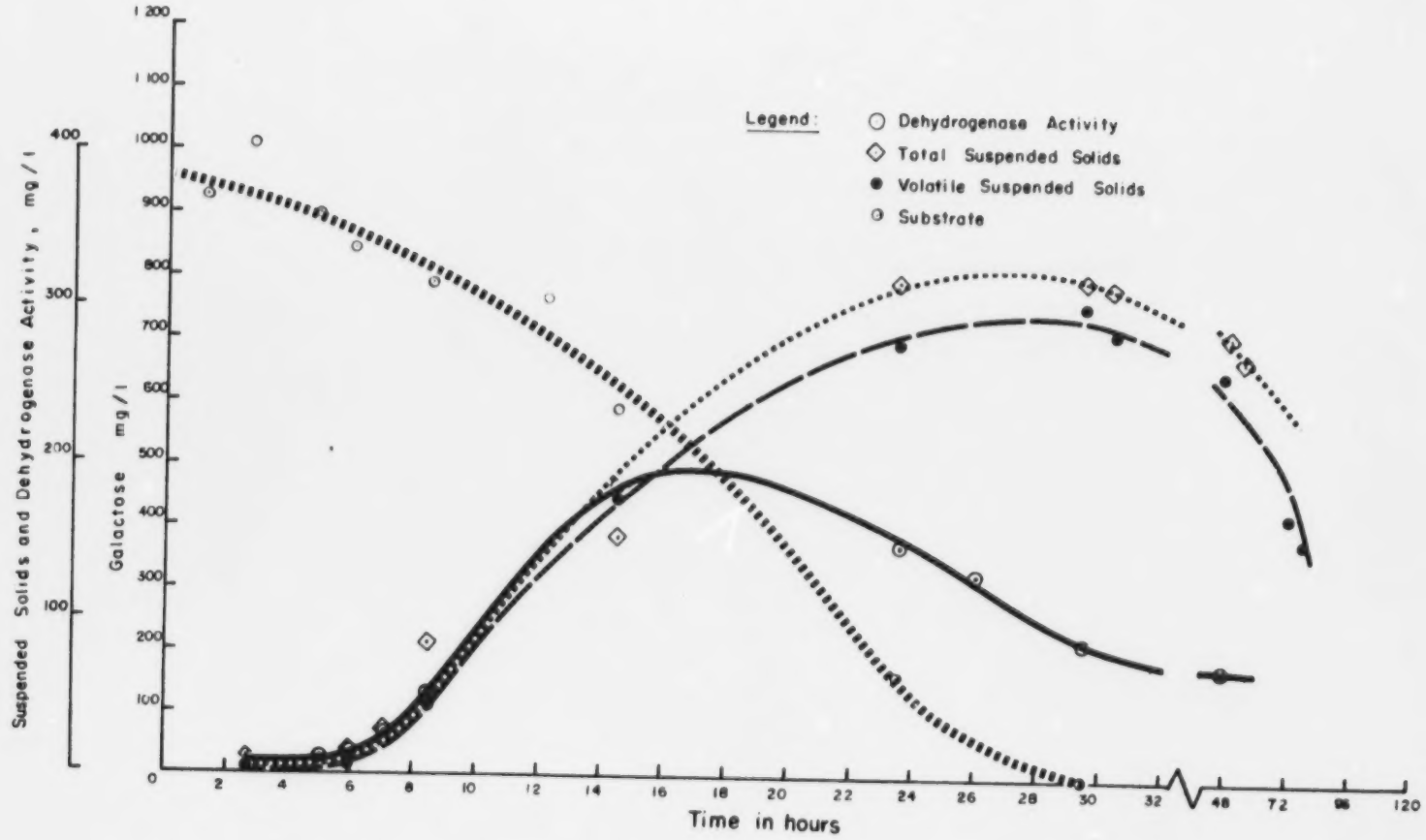

Figure 23. Heterogeneous Culture Batch No. 4 With Galactose Substrate

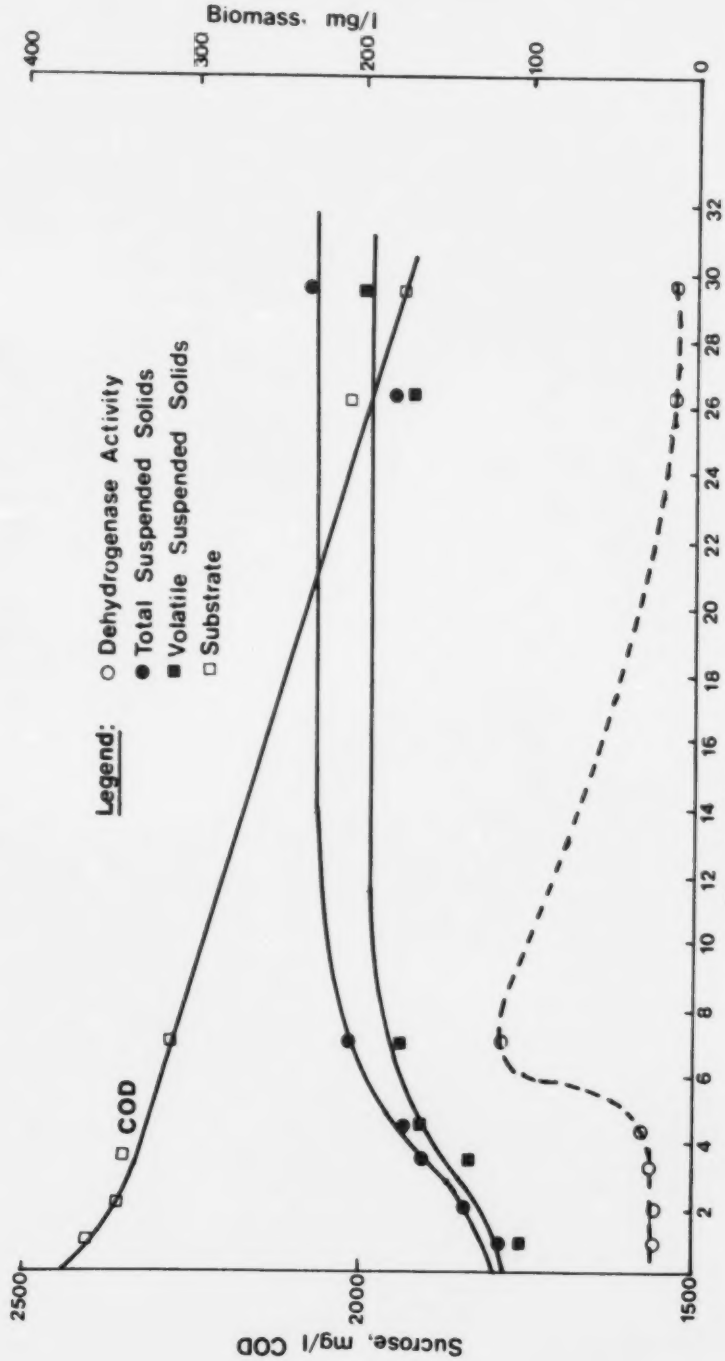

Figure 24. Heterogeneous Culture Batch No. 5 With Sucrose Substrate

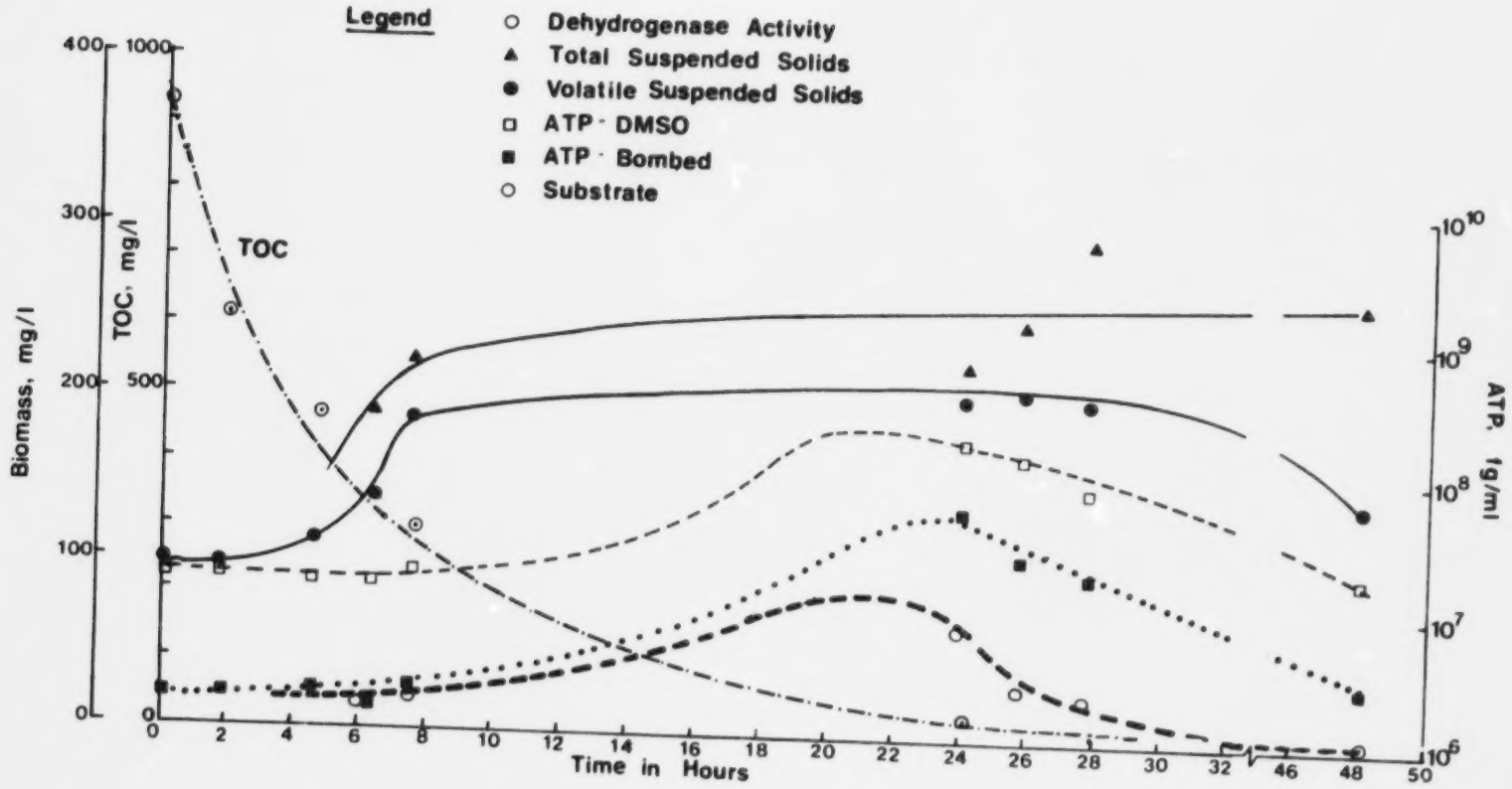

Figure 25. Heterogeneous Culture Batch No. 6 With Acetic Acid Substrate

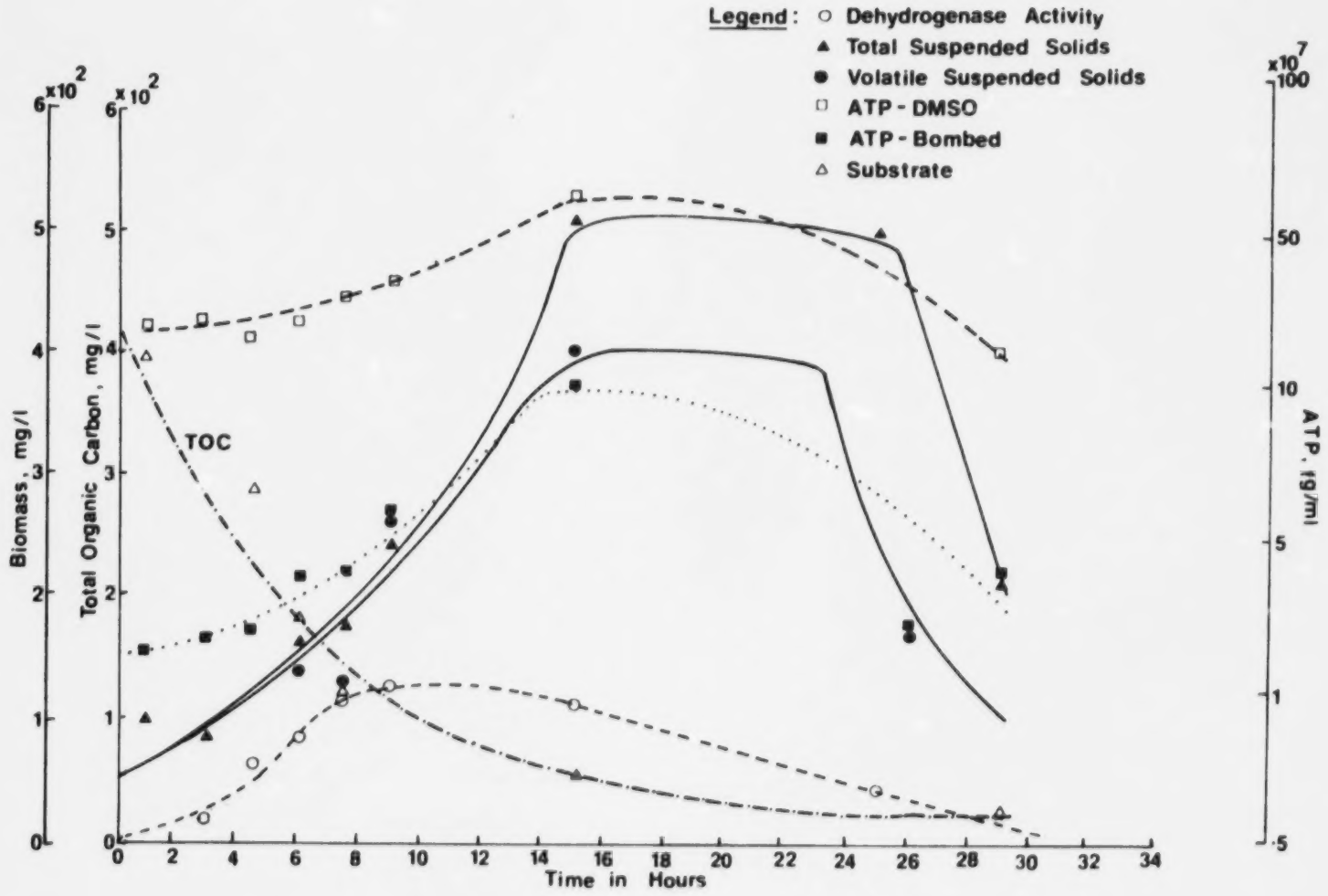

Figure 26. Heterogeneous Culture Batch No. 7 With L-Alanine Substrate

TABLE 23

Heterogeneous Anaerobic Culture Batch No. 1 With Leachate

Time, hour	Leachate by COD, mg/l	Biomass Concentrations By			Total Gas Production, ml
		Dehydrogenase Activity, mg/l	Total Suspended Solids, mg/l	Volatile Suspended Solids, mg/l	
0		27.9	990	490	0
0.25	330	84.8	810	430	0
2.50	307	91.6	730	395	70
4.25	279	64.1	720	355	160
6.00	250	61.3	735	370	270
8.00	213	-	725	360	410
14.25	160	-	805	420	680
25.00	133	62.0	740	375	870

TABLE 24

Heterogeneous Anaerobic Culture Batch No. 2 With Leachate

Time, hour	Leachate by COD, mg/l	Biomass Concentrations By					Total Gas Production, ml
		Dehydrogenase Activity, mg/l	Total Suspended Solids, mg/l	Volatile Suspended Solids, mg/l	ATP, fg/ml		
					DMSO	Bombing	
0	538	45.5	525	320	2.55×10^7	9.40×10^6	0
1.00	-	51.7	490	275	1.80×10^7	6.22×10^5	30
2.50	518	44.4	470	270	1.35×10^7	5.32×10^5	90
4.00	489	34.4	460	275	1.24×10^7	6.33×10^5	160
5.25	-	-	455	310	8.76×10^6	4.58×10^5	200
9.50	446	-	550	330	8.64×10^6	3.71×10^5	420
19.50	363	25.5	490	310	9.18×10^6	5.2×10^5	800
23.50	326	25.5	525	335	7.47×10^6	6.57×10^5	900
27.50			515	295	6.51×10^6	5.34×10^5	980

TABLE 25

Heterogeneous Anaerobic Culture Batch No. 3 With Leachate

Time, hour	Leachate by COD, mg/l	Biomass Concentrations By					Total Gas Production, ml
		Dehydrogenase Activity, mg/l	Total Suspended Solids, mg/l	Volatile Suspended Solids, mg/l	ATP By		
					DMSO	Bombing	
0	774	9.4	3,190	555	3.45×10^7	1.48×10^6	0
1.5	738	25.8	2,990	550	1.5×10^7	1.07×10^6	40
3.0	-	42.9	3,405	590	1.27×10^7	7.11×10^5	180
4.5	760	47.6	3,380	575	9.60×10^6	1.24×10^6	280
6.0	741	32.1	3,220	555	8.46×10^6	1.64×10^6	360
7.5	723	43.3	3,350	565	8.52×10^6	5.03×10^5	490
9.0	701	-	3,170	580	9.48×10^6	1.13×10^6	570
13.5	647	8.1	3,100	615	7.26×10^6	6.33×10^5	780
24.0	621	7.9	2,930	585	7.56×10^6	8.63×10^5	1,020
54.0			2,775	515	5.04×10^6	3.62×10^5	1,210

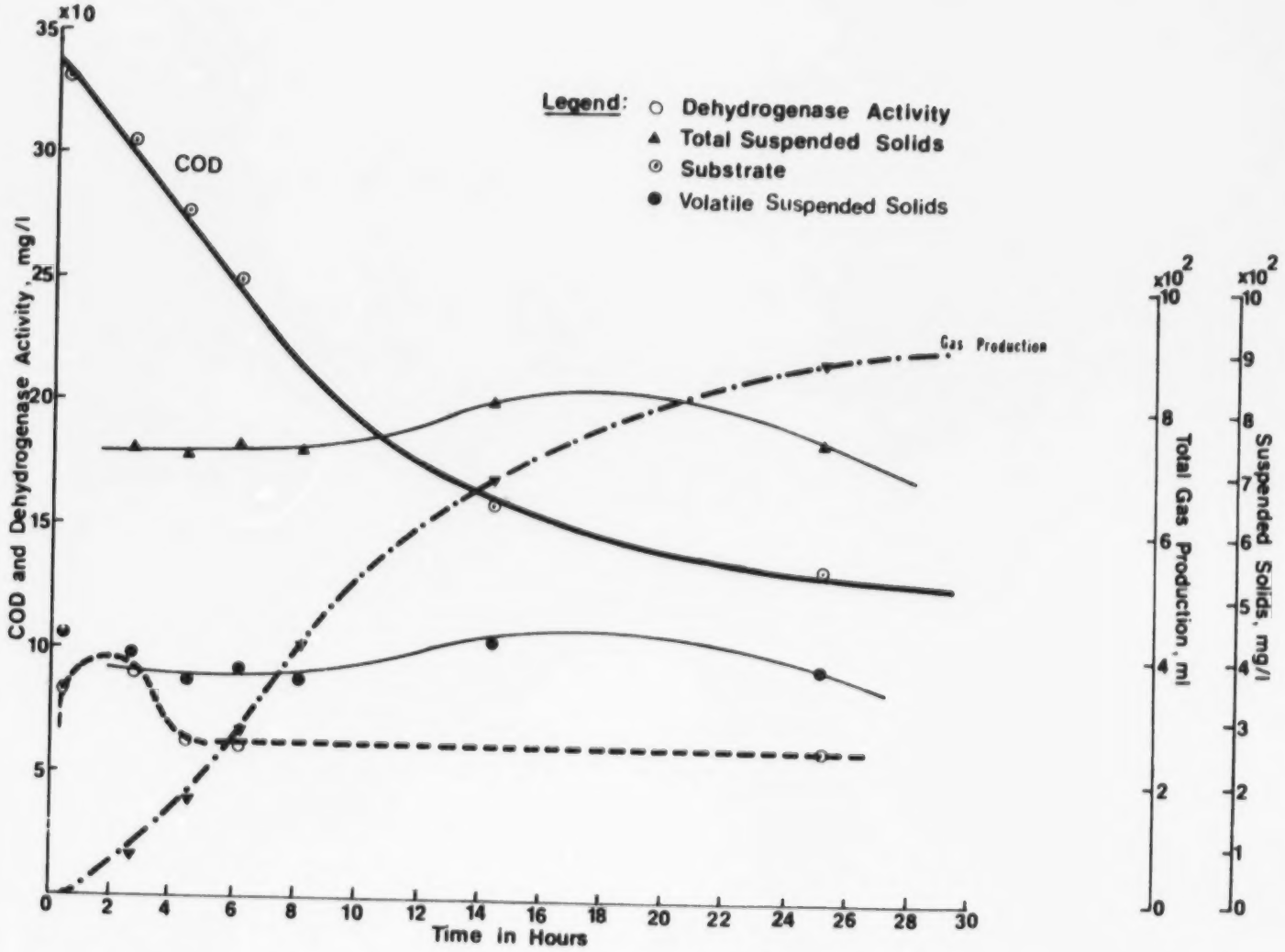

Figure 27. Heterogeneous Anaerobic Culture Batch No. 1 With Leachate

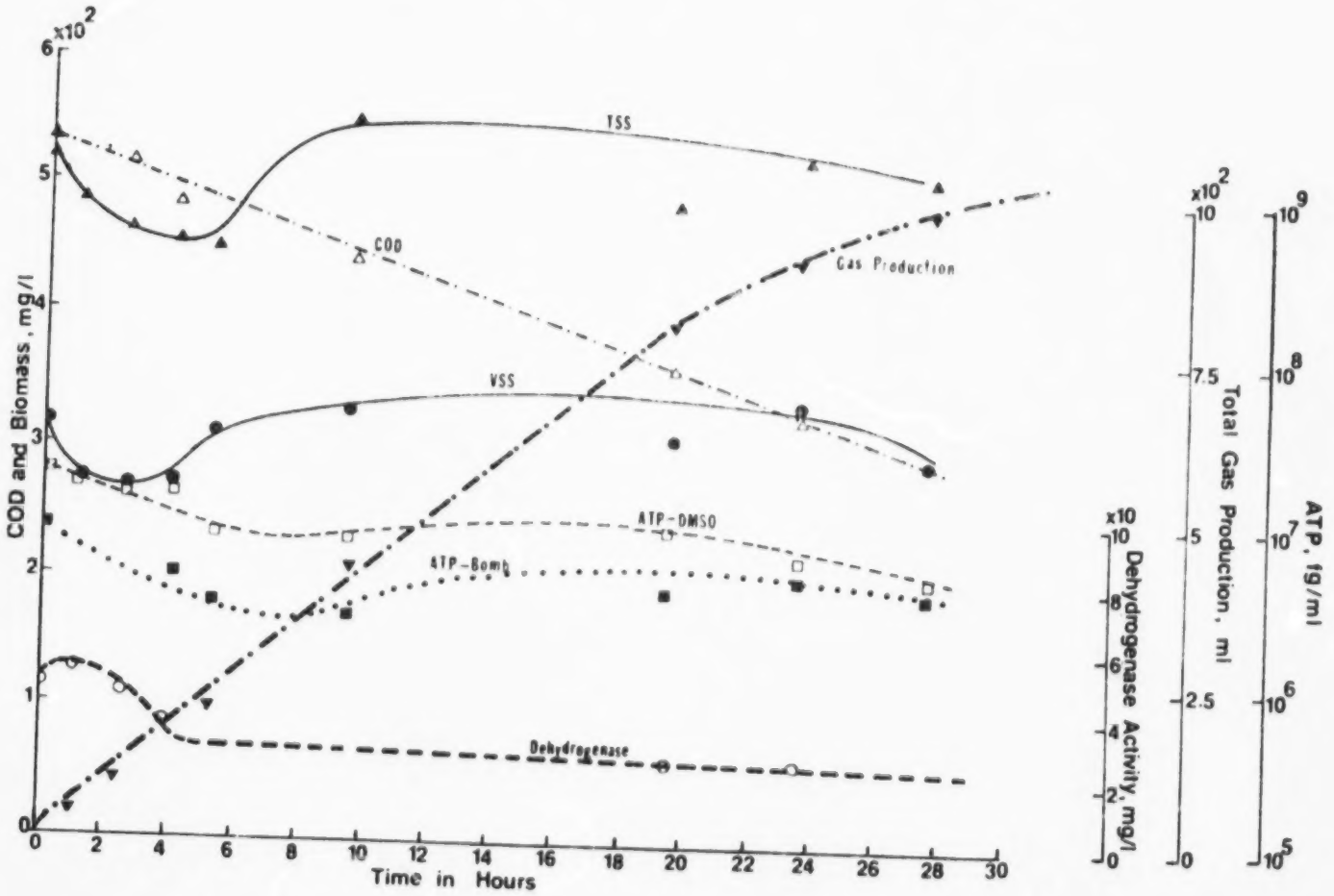

Figure 28. Heterogeneous Anaerobic Culture Batch No. 2 With Leachate

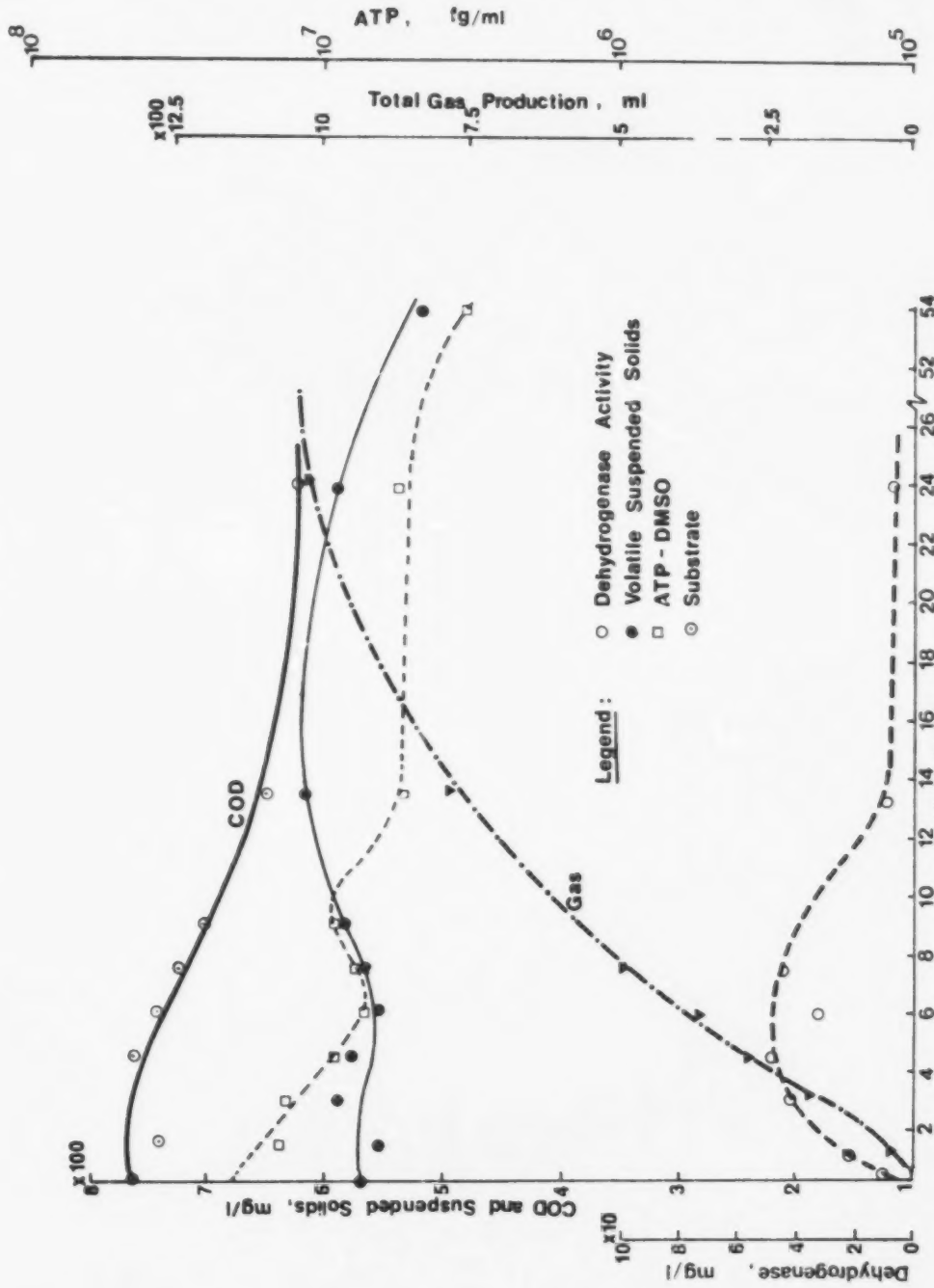

Figure 29. Heterogeneous Anaerobic Culture Batch No. 3 With Leachate

TABLE 26

Ratios Between Biomass Parameters during Log Growth Phase of Batch Cultures

Substrate	Dehydrogenase / VSS	Plate Count / Coulter Counter	Culture
Glucose	0.60 - 0.70	0.82	Pure culture with *E. coli*
Galactose	0.45 - 0.6	0.35 - 0.40	"
Sucrose	0.66	0.42	"
Acetic Acid	0.60	0.41	"
Alanine	0.70	0.78	"
Glucose	0.66	-	Heterogeneous culture
Galactose	0.90	-	"
Sucrose	0.60	-	"
Acetic Acid	0.40	-	"
Alanine	0.60	-	"

Continuous Culture Studies:

The series of continuous culture experiments were continued to study the application of dehydrogenase activity and ATP measurements under steady state conditions. Steady state was established by operating the reactors for periods of 3 to 4 retention times prior to sampling and analysis for each of the substrates. The results are included in Tables 27-32 and Figures 30-35. The substrates used during the studies included not only simple sugars like glucose (in aerobic and anaerobic) and galactose but also industrial wastes from shellfish and chicken processing plants and leachate from a solid waste disposal site. Parameters monitored in these studies were similar to those used during the batch culture studies. COD, 5-day biochemical oxygen demand (BOD_5) and/or total organic cabon (TOC) were used to measure the substrate concentrations for these latter indistrial wastes.

The data on biomass measurement indicated similarity between parameters during the steady state observations. The observed ratio of the dehydrogenase activity to suspended solids remained fairly constant and close to unity with the glucose and galactose substrate in the aerobic cultures and also constant but lower (0.70) in the anaerobic cultures except when very long retention times were investigated. It was further observed that this ratio was 0.35 on chicken processing wastes and 0.60 on shellfish processing wastes as shown in Table 33. Since the dehydrogenase activity was reported decreasing in the endogenous growth phase during batch culture, it become self-explanatory that the preceding ratio would decrease at very long retention times where partial endogenous growth existed. It became more evident when this ratio decreased to 0.30 with the same galactose substrate in aerobic cultures and only the sludges were being recycled as presented in the succeeding section of this report. Unlike the correlations between ATP, organic nitrogen, or other parameters and VSS varing with the specific growth rates, it is very important to observe the consistent correlation between the dehydrogenase activity and VSS with a given substrate in continuous cultures.

The different organic character of the substrate resulted in changing ratios of dehydrogenase activity to weight of solids as indicated for the selected industrial wastewaters. It is noted that not all the volatile solids reported represented biological mass. The correlation established from the simple sugar substrate studies should be different from those for the industrial wastes investigated here. By plotting data from studies on chicken and shellfish processing wastes the following emperical equation was obtained.

$$X = 930\ A + 10 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (2)$$

where: X = active biomass, mg/l

A = dehydrogenase activity measured as absorbance at 483 mμ and 1 cm light path

From these data it follows that once the correlating ratio between the parameters have been established in an actual waste treatment process the active biomass could be monitored more accurately and more rapid corrective measures taken as problems develop.

Kinetic constants for all studies were calculated and compared in Table 34. The maximum specific growth rate was higher on glucose-grown cultures than on the other sugars, amin acids or industrial wastes.

Continuous Culture Studies with Solids Recycle: A continuous culture study with solids recycle was conducted with a galactose substrate as shown in Table 35 and Figures 36 and 37. These results indicated that the steady state galactose concentrations were much lower than those observed in the same system without recycle. The biomass concentrations as measured by VSS, dehydrogenase activity and ATP at shorter retention times were observed almost doubled as the recycle factor increased from 1.6 to 5.8 and inversely porportional to the settling time in the clarifier. Increased rates of substrate utilization with recycle were attributed to opportunitites for more rapid growth and the magnitude of biomass concentration by recyle particularly at shorter retention times.

To determine changes in active biomass with changes in specific growth rate, dehydrogenase and ATP vs. VSS were plotted as shown on Figure 38. These data are included in Table 36 and indicated that the ATP content in the solids rapidly increased and then decreased gradually with the increase of growth rate. At low specific growth rates (up to 1.5 day^{-1}), ATP content increased to 0.45 mg ATP per gram VSS while at high specific growth rates, ATP content decreased to a limiting range of 0.25 - 0.27 mg ATP per gram VSS and finally decreased when organism washout occurred. The cause of this rapid increase followed by a decrease has not been well established. However, the same behavoir was illustrated also by the data of Weddle and Jenkins [3] on activated sludge although this early increase was essentially ignored in their analysis. The ATP content of pure cultures have been reported varying from 0.02 to 1.2 percent on a dry weight bases [35,39], while those for activated sludge of 0.2 and 0.3 mg ATP/g dry weight by Patterson, et al.[2] and up to 2.0 mg ATP/g ss by Biospherics[54] are in good agreement with the data obtained from the study with solids recycle. The initital rapid increase of ATP content per weight of VSS could reflect more growth due to the sufficiently high availability of substrates which may not have been possible at longer retention times. The decrease of ATP appeared to have resulted from either washout of certain organisms of high ATP content or a smaller capacity for ATP storage inside the cells when organisms grow faster by utilizing more energy at higher growth rates. Therefore, the correlation between ATP and VSS could be meaningfully applied for control of continuous culture type systems within the growth ranges where essentially not much change occurred (above 2.0 day^{-1}).

The dehydrogenase activity increased steadily with growth rate (to a specific growth rate of 3 day^{-1}) and then remained essentially constant until washout occurred. However, the ratio between the dehydrogenase and VSS indicated virtually no sifnificant change throughout the range of specific growth rates covered, even though it was recognized that the overall 0.30 level was only one third of that from the study without solids recycle (Table 28). The existence of a partial

endogenous growth phase by solids recycle was considered the major cause of a decreased ratio. Generally constant nature of this correlation between dehydrogenase activity per weight of biomass permits the determination of active biomass concentrations in cultures operating at any practical growth rate by establishing a standard curve correlating these parameters at exponential growth.

TABLE 27

Continuous Culture Study with Glucose Substrate

Retension Time, hr.	Glucose mg/l	Biomass Concentrations		
		Dehydrogenase Activity, mg/l	Total Suspended Solids, mg/l	Volatile Suspended Solids, mg/l
24.0	0.3	89.9	115	100
18.5	0.4	-	125.0 127.5	110 115
15.5	-	121.2 140.0	120 150	110 115
12.3	6.0	111.6 135.5	97.5 105	80
6.0	14.7	71.5	65	65
4.0	70.7	33.0	32	32

TABLE 28

Continuous Culture Study with Galactose Substrate

Retention Time, Hrs.	Galactose, mg/l	Biomass Concentrations	
		Dehydrogenase Activity, mg/l	Total Suspended Solids, mg/l
5.92	4.3	83.5	90.0
5.83	4.0	63.9	66.0
3.81	1.9	92.5	102.0
2.96	87.7	40.0	60.0
2.84	26.8	98.8	100.0
2.06	24.0	53.9	63.0
1.65	133.9	54.5	53.1

TABLE 29

Continuous Culture Study with Shellfish Processing Wastes

Retention Time, Hrs.	Substrate by BOD_5, mg/l	Biomass Concentrations By			
		Dehydrogenase Activity mg/l	Total Suspended Solids, mg/l	ATP, fg/ml	
				DMSO X 10^6	Bombing X 10^6
12	50.0	-	88	9.1	8.2
10	33.3	41.7	84	26.0	4.1
8	-	58.0	83	36.0	10.0
6	47.6	-	-	-	-
4	95.1	66.7	102	49.2	7.4
2	166.1	8.8	94	17.5	5.1

TABLE 30

Continuous Culture Study with Chicken Processing Wastes

Retention Time, Hrs.	Substrate		Biomass Concentrations By			
	BOD_5, mg/l	COD, mg/l	Dehydrogenase Activity, mg/l	Total Suspended Solids, mg/l	ATP, fg/ml	
					DMSO	Bombing
20	116	157	54.6	193	5.60×10^7	1.26×10^7
16	---	---	---	220	---	---
11	118	165	30.7	116	3.59×10^7	3.75×10^6
8	130	---	---	150	9.18×10^6	2.14×10^5
6	125	185	69.9	176	5.21×10^7	2.78×10^6
2	160	260	45.6	75	3.79×10^7	5.42×10^6

TABLE 31

Continuous Culture Study with Leachate

Retention Time, Hrs.	Leachate by		Biomass Concentrations by			
	TOC, mg/l	COD, mg/l	Dehydrogenase Activity, mg/l	Total Suspended Solids, mg/l	Volatile Suspended Solids, mg/l	ATP, fg/ml
15	273	700	495	3116	1993	7.6×10^7
10	307	674	672	3450	2040	---
5	385	850	1064	5470	2940	1.53×10^8
2	596	1860	---	3150	1235	1.99×10^7

TABLE 32

Anaerobic Digester with Heterogeneous Cultures
in Continuous Flow System

Retention Time, Hr.	Glucose mg/l	Biomass Concentrations by		
		Dehydrogenase Activity, mg/l	Total Suspended Solids, mg/l	Volatile Suspended Solids, mg/l
44.7	1.0	154.9	384	110
28.7	2.5	219.4	458	270
26.5	5.0	250.0	384	180
20.0	14.0	300.0	355	173
16.6	15.3	284.0	365	177
8.8	5.3	347.0	307	166
6.0	10.6	116.0	279	132
4.0	194.0	93.6	235	95

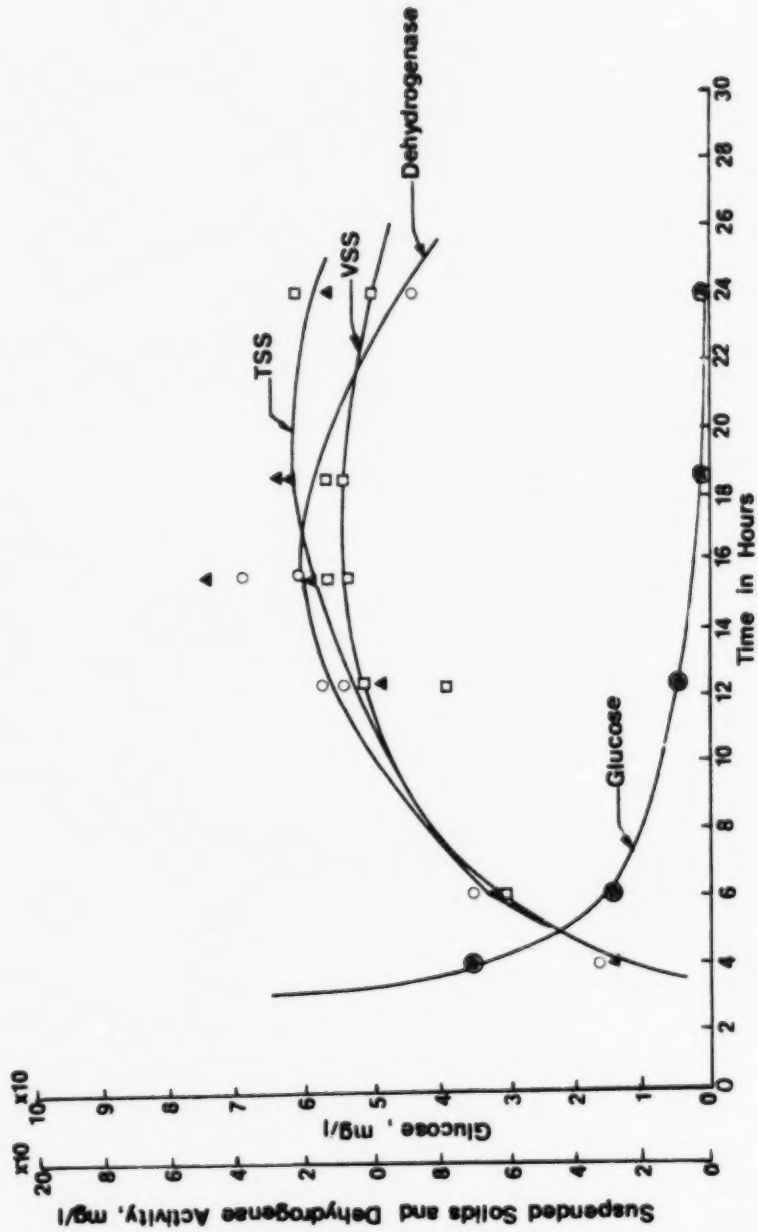

Figure 30. Continuous Culture Study With Glucose Substrate

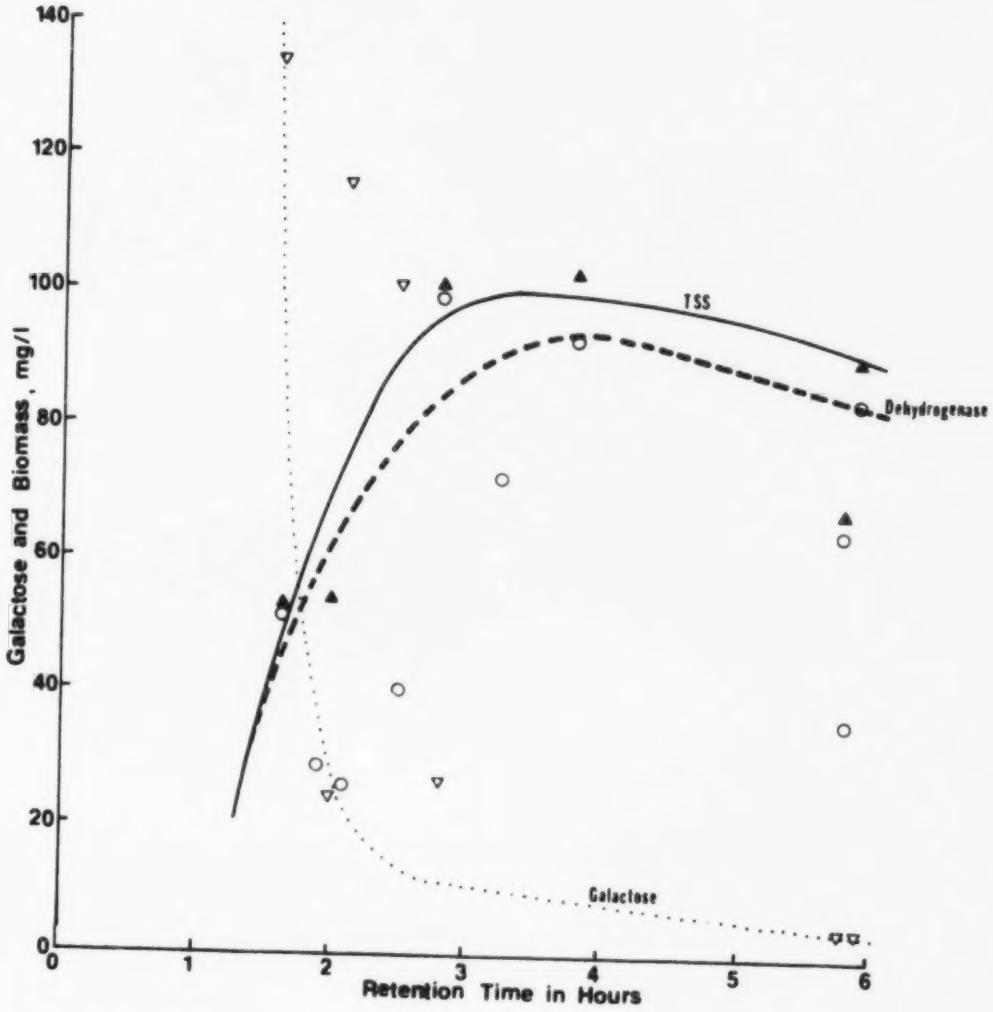

Figure 31. Continuous Culture Study With Galactose

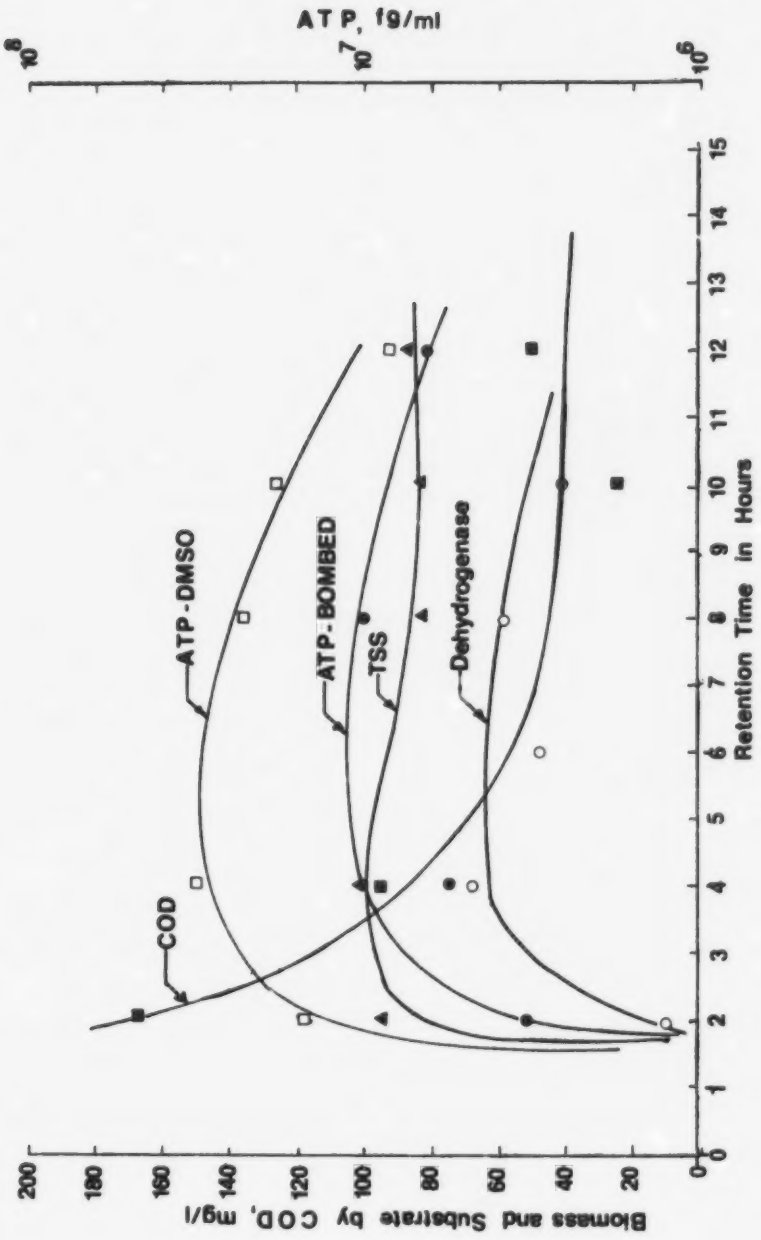

Figure 32. Continuous Culture Study With Shellfish Processing Wastes

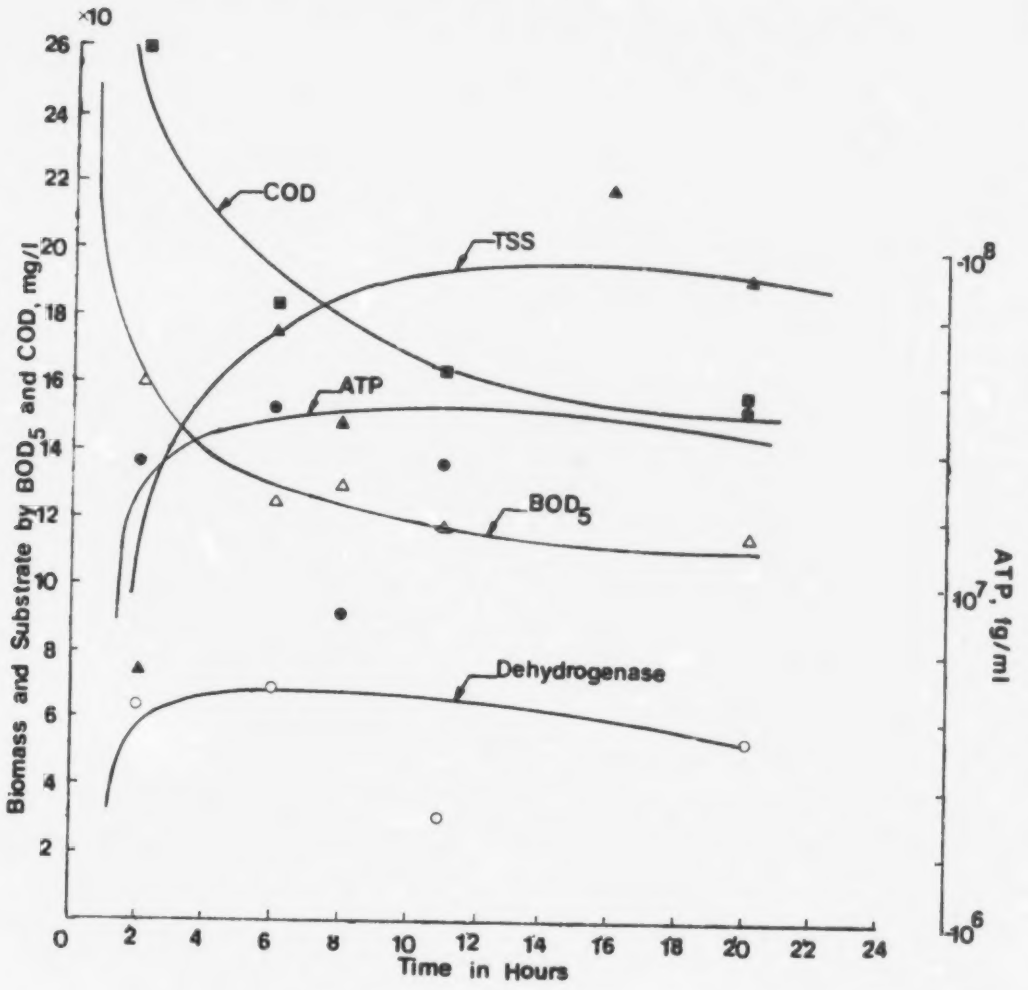

Figure 33. Continuous Culture Study With Chicken Processing Waste

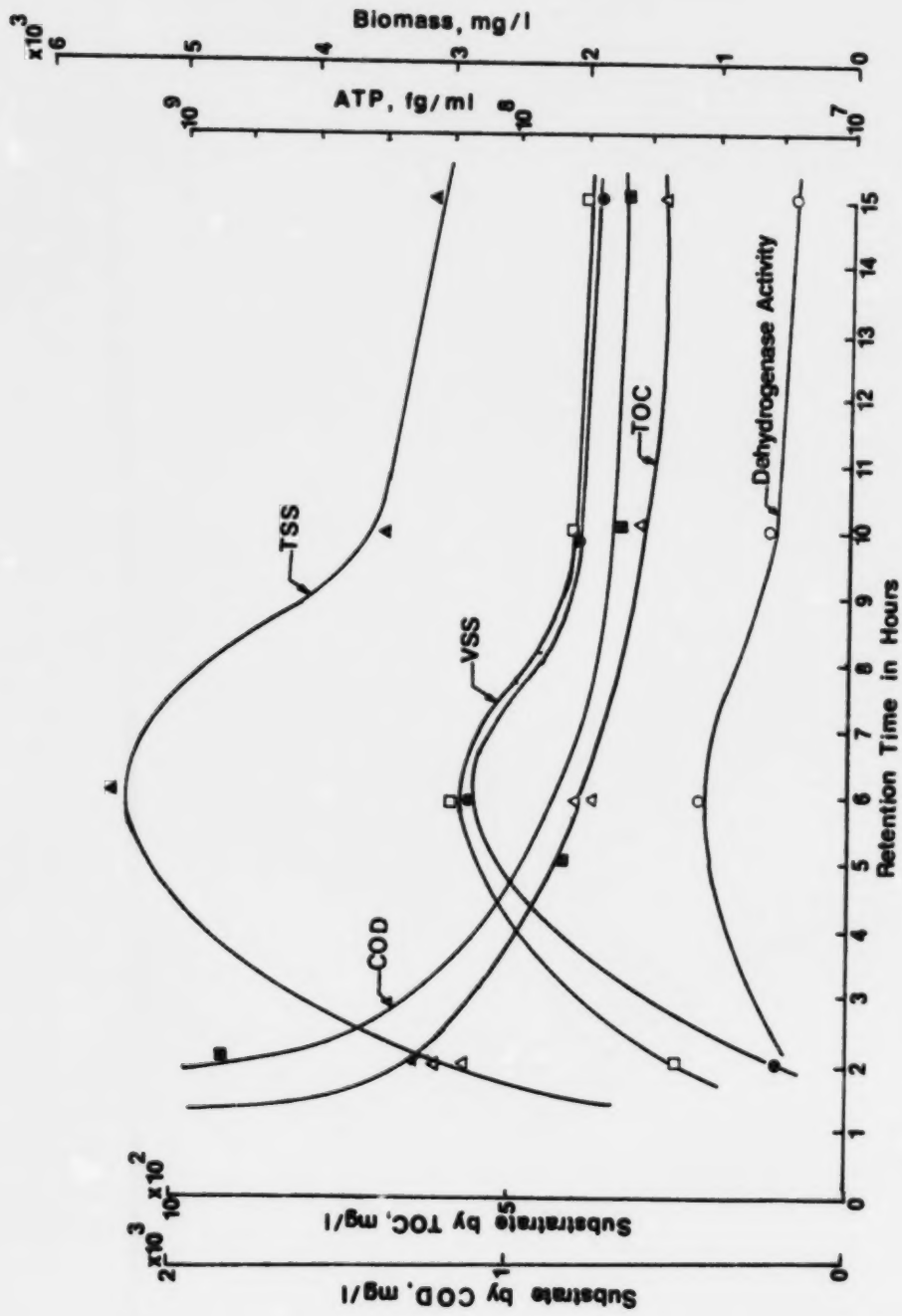

Figure 34. Continuous Culture Study With Leachate

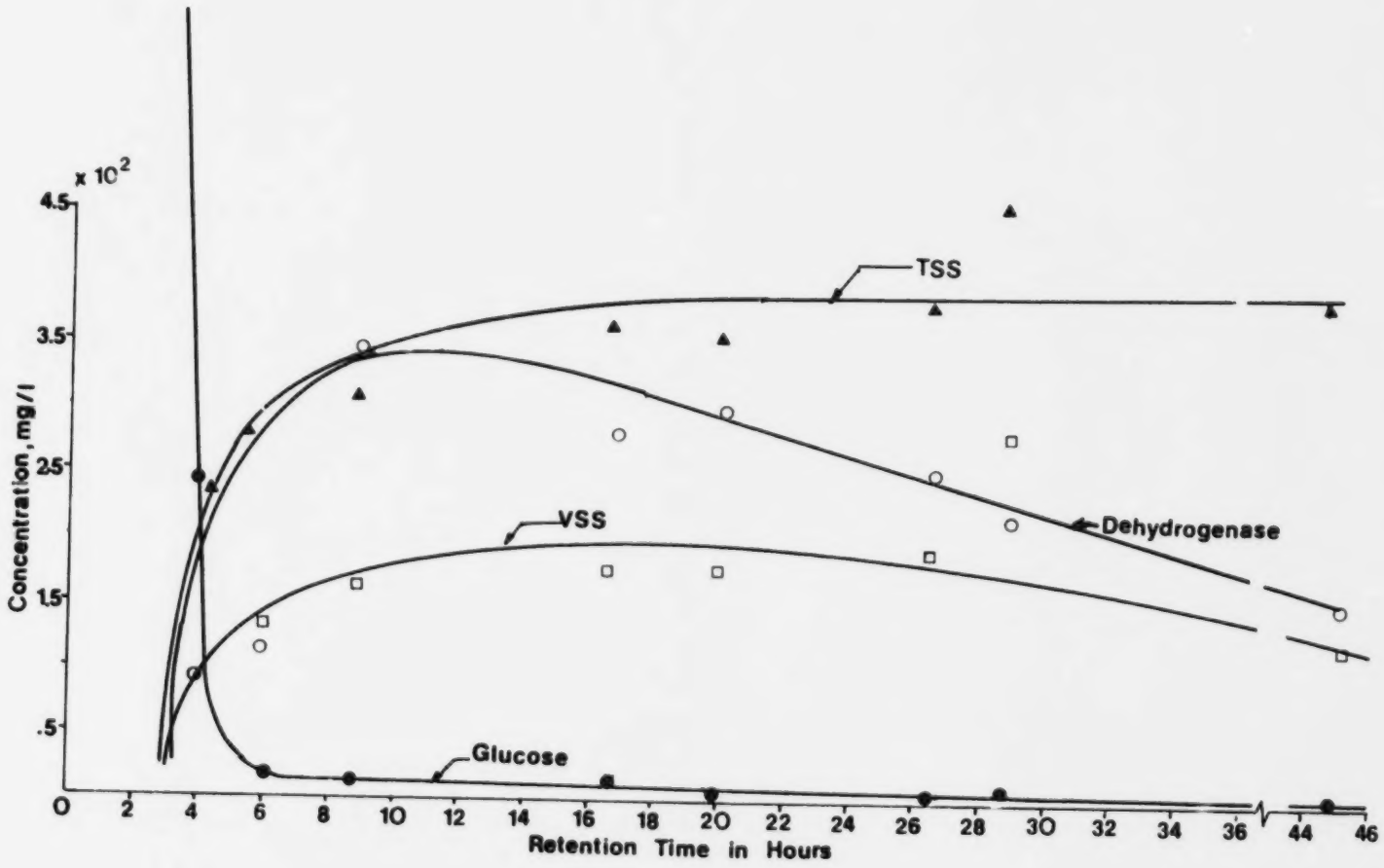

Figure 35. Anaerobic Digester With Heterogeneous Populations in Continuous Flow System

TABLE 33

Summary of Correlations between Biomass Parameters
in Continuous Culture Studies

Substrates	$\frac{\text{Dehydrogenase}}{\text{VSS}}$	$\frac{\mu g \text{ ATP}}{mg \text{ VSS}}$	Remarks
glucose	0.8 ~ 1.0	-	aerobic cultures
galactose	0.9 ~ 1.0	-	"
chicken waste	0.3 ~ 0.4	0.27 ~ 0.29	"
shellfish waste	0.5 ~ 0.6	0.30 ~ 0.63	"
galactose with Recycle	0.2 ~ 0.3	0.26 ~ 0.46	"
glucose	0.6 ~ 0.8	-	anaerobic cultures

TABLE 34

Kinetic Growth Constants

Experiment		Substrate	Maximum Specific Growth Rate, $hour^{-1}$	Saturation Constant, mg/l
Pure Culture	No. 1	Glucose	0.040	118.2
Pure Culture	No. 2	Glucose	0.079	416.0
Pure Culture	No. 3	Glucose	0.056	538.0
Pure Culture	No. 5	Galactose	0.037	96.9
Pure Culture	No. 6	Galactose	0.025	181.0
Pure Culture	No. 8	Acetic Acid	0.021	1,165.0
Pure Culture	No. 9	Acetic Acid	0.017	4,230.0
Heterogeneous Culture	No. 1	Glucose	0.457	995.0
Heterogeneous Culture	No. 2	Glucose	0.328	2,460.0
Heterogeneous Culture	No. 3	Galactose	0.141	150.5
Heterogeneous Culture	No. 4	Galactose	0.098	146.0
Continuous Culture		Glucose	0.625	4.1
Continuous Culture		Shellfish Waste	0.40	45.0*
Continuous Culture		Leachate	1.0	1,460 **
Continuous Culture		Galactose	0.46	5.7
Anaerobic Digester		Glucose	0.435	19.60

* BOD_5 basis

** COD basis

TABLE 35

Continuous Culture Study with Galactose Substrate and Solids Recycle

Retention Time, hour	Galactose, mg/l	Biomass Concentrations By					
		Dehydrogenase Activity, mg/l		Volatile Suspended Solids, mg/l		ATP, x 10^8, fg/ml	
		Reactor	Clarifier Effluent	Reactor	Clarifier Effluent	Reactor	Clarifier Effluent
11.9	2.6	114.5	43.7	655	400	1.91	1.25
6.0	3.4	141.0	23.8	570	195	2.62	1.84
2.5	1.2	212.0	11.2	770	210	2.29	1.30
1.25	10.4	445.0	32.8	1,270	220	3.58	1.34
0.80	405.0	249.0	19.0	840	200	2.16	0.70

TABLE 36

Summary of Growth Constants and Ratios
Between Parameters in Solids Recycle Study
with Galactose Substrate

Retention Time (hour)	Recycle Factor*	Specific** Growth Rate, day^{-1}	ATP vs. VSS, $\frac{mg\ ATP}{g\ VSS}$	Dehydrogenase Activity vs. VSS
11.90	1.64	1.22	0.292	0.18
6.00	2.92	1.37	0.457	0.24
2.50	3.67	2.46	0.377	0.28
1.25	5.77	3.23	0.282	0.35
0.80	4.20	7.22	0.257	0.30

* Ratio of VSS in reactor to the VSS in clarifier effluent.

** $\mu = \frac{Q}{V} \cdot \frac{1}{(R.F.)}$

where μ = specific growth rate, day^{-1}

Q = influent, l/day

V = volume, l

R.F. = Recycle Factor

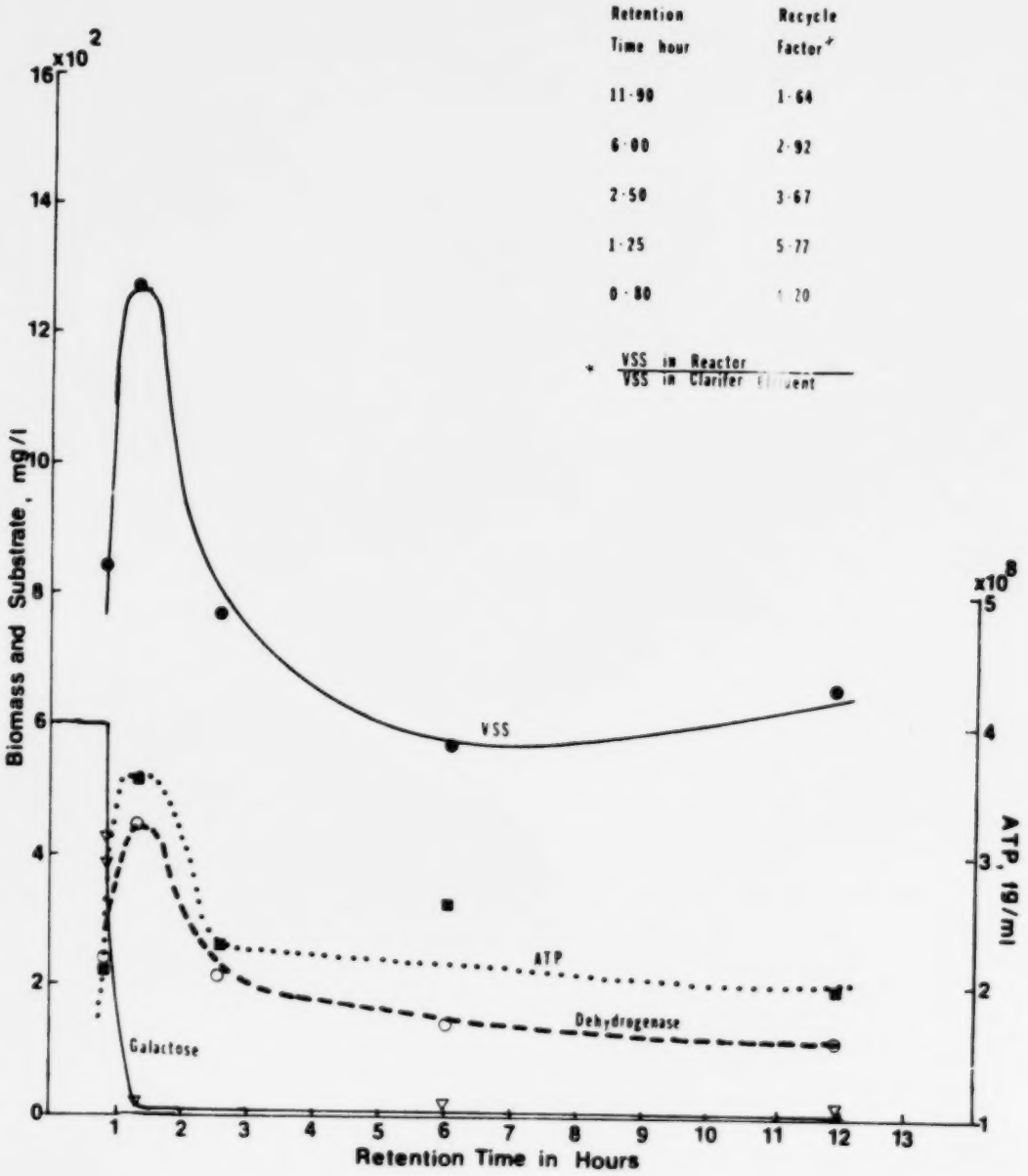

Figure 36. Continuous Culture Study With Galactose Substrate and With Solids Recycle

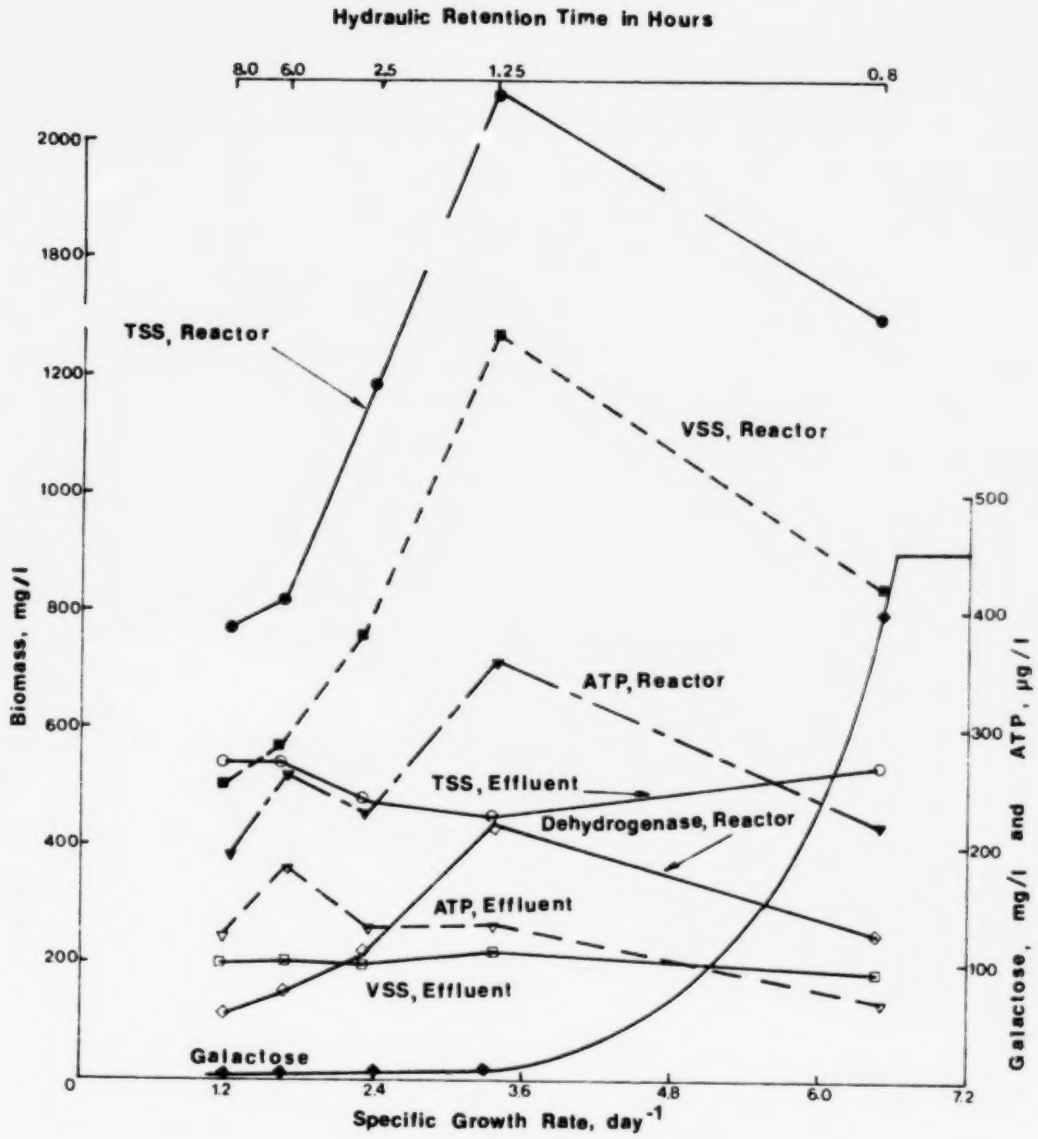

Figure 37. Active Biomass Measurements in Continuous Culture Study With Solids Recycle

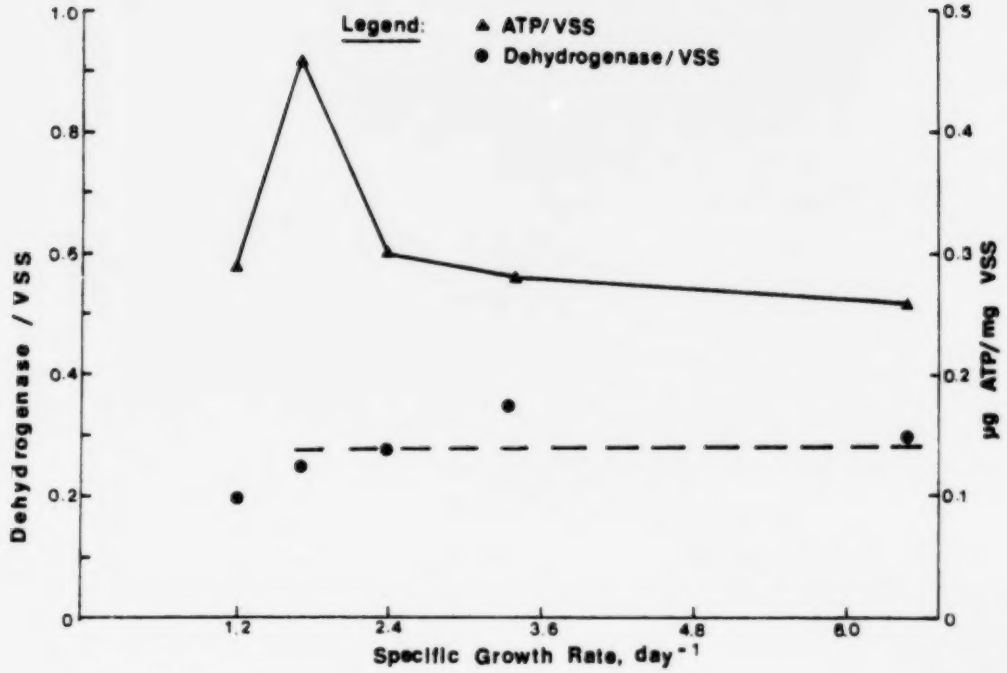

Figure 38. Effect of Specific Growth Rate on Correlations Between Biomass Measurements in Continuous Culture Study With Recycle

Nutrient Deficiency Studies - Heterogeneous batch cultures on media deficient in nitrogen or phosphorus were grown on glucose as the sole carbon and energy source in order to investigate the effect on the dehydrogenase actibity. In the nitrogen deficiency study, the ratios of carbon to nitrogen were selected at 10, 20 and 30 to successive batches (nos. 1, 2 & 3) and the results are shown in Tables 37,38 and 39 and Figures 39, 40 and 41. Carbon to phosphorus ratios of 150 and 200 were selected in phosphorus deficiency studies. No measurable growth was observed in the latter and the results, of the former (Batch No. 4) are shown in Table 40 and Figure 42.

It was observed that the dehydrogenase activity was again a very sensitive indicator of the biomass and the same correlation was established with VSS, except with cultures grown on extreme nitrogen and phosphorus deficiencies, i.e., when C/N was 30 and C/P was 150. However, the ATP data indicated good agreement with VSS even during extreme deficiencies. For instance, about 75 percent of the maximum ATP and VSS in Batch No. 1 (Table 41) was observed in Batch No. 3 while only a third of the dehydrogenase activities was indicated. Therefore, it could be deduced that the dehydrogenase activity was limited in application under extreme nutrient deficiency, while the ATP measurement was acceptable even under this condidion. Since the carbon to nitrogen ratio in domestic sewage normally does not indicate such a deficiency, the application of the dehydrogenase test remains viable.

TABLE 37

Nutrient Deficient Culture Batch No. 1 (C/N=10)

Time, hour	Glucose, mg/l	Biomass By		
		Dehydrogenase Activity, mg/l	Volatile Suspended Solids, mg/l	ATP x 10^7, fg/ml
0	1,000			
1.0	1,000	0	40	6.76
4.5	-	122.9	-	8.37
6.0	541	211.4	250	12.10
7.0	102	227	220	13.10
8.0	15	-	280	11.10
13.0	0	223	290	15.30
24.0	0	10	105	8.30
28.5	0	0	-	3.99
30.5	0	0	50	1.70

TABLE 38

Nutrient Deficient Culture Batch No. 2, (C/N = 20)

Time, hours	Glucose, mg/l	Biomass by		
		Dehydrogenase Activity, mg/l	Volatile Suspended Solids, mg/l	ATP x 10^7, fg/ml
0	1025	-	52	-
1.0	972	8.5	68	6.72
2.5	863	104.0	92	-
4.5	580	210.0	200	12.30
6.3	488	185.0	190	15.10
7.5	399	222.0	185	26.70
14.5	0	205.0	280	-
24.0	0	71.0	280	13.40
29.3	0	84.8	255	8.88
53.3	0	-	185	5.43

TABLE 39

Nutrient Deficient Culture Batch No. 3. (C/N = 30)

Time, hours	Glucose, mg/l	Biomass by		
		Dehydrogenase Activity, mg/l	Volatile Suspended Solids, mg/l	ATP $\times 10^7$, fg/ml
1.0	975	-	20	1.80
2.0	988	-	30	2.27
3.3	940	9.1	26	2.43
5.3	1000	19.4	36	6.12
6.5	700	39.5	83.5	8.84
7.5	525	57.8	116.0	5.98
8.3	412	-	180.0	5.98
12.3	84	66.7	200.0	11.30
22.5	0	35.5	180.0	9.32
25.0	0	36.9	185.0	7.93
28.8	0	40.8	185.0	7.28
52.3	0	16.0	225	8.02

TABLE 40

Nutrient Deficient Culture Batch No. 4 (C/P = 150)

Time, hours	Glucose, mg/l	Biomass by		
		Dehydrogenase Activity, mg/l	Volatile Suspended Solids, mg/l	ATP x 10^7, fg/ml
0	826	-	-	-
2.0	806	6.8	25	1.08
4.0	813	7.9	50	1.26
6.0	780	18.8	60	1.87
7.5	767	13.9	55	2.36
11.5	737	-	65	3.08
24.5	687	-	75	2.81
29.0	670	-	65	2.64

TABLE 41

Comparison of Biomass from Nutritional Deficiency Culture Studies

Biomass By	Batch No.			
	1	2	3	4
	C/N=10	C/N=20	C/N=30	C/P=150
Max. VSS, mg/l	290	280	200	75
Max. Dehydrogenase, mg/l	227	200	70	19
Max. ATP, mg/l	150	134	114	31

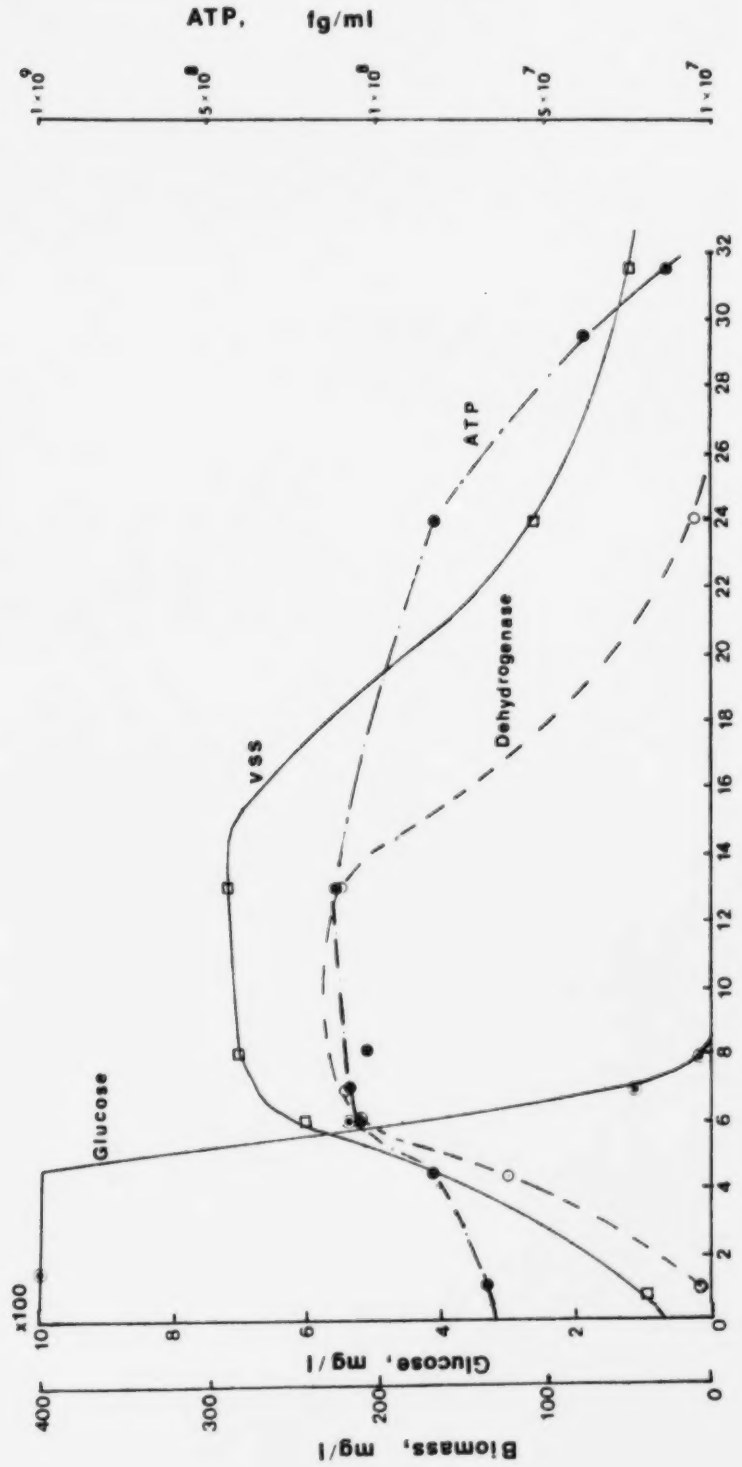

Figure 39. Nutrient Deficient Culture Batch No. 1 (C/N=10)

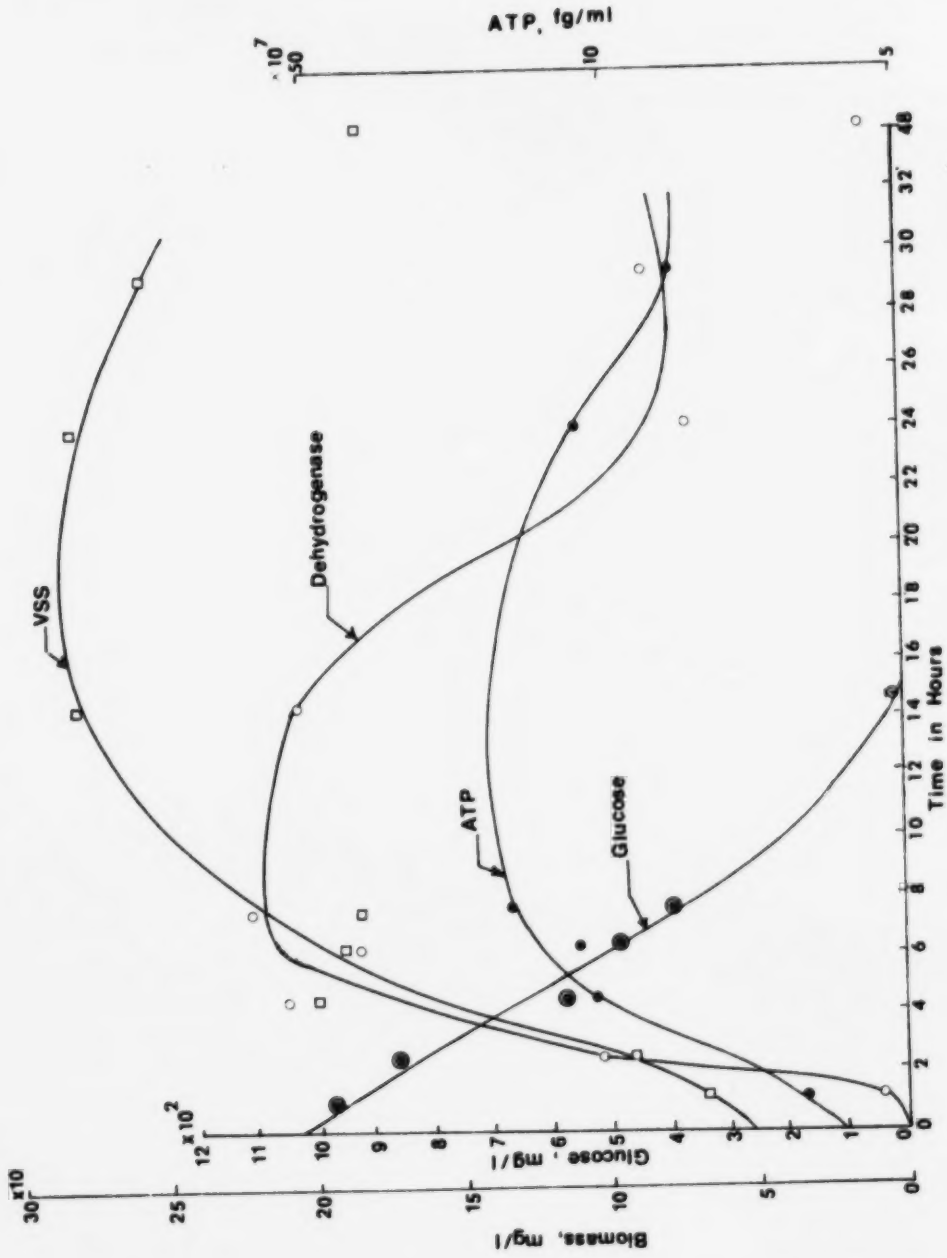

Figure 40. Nutrient Deficient Culture Batch No. 2 (C/N=20)

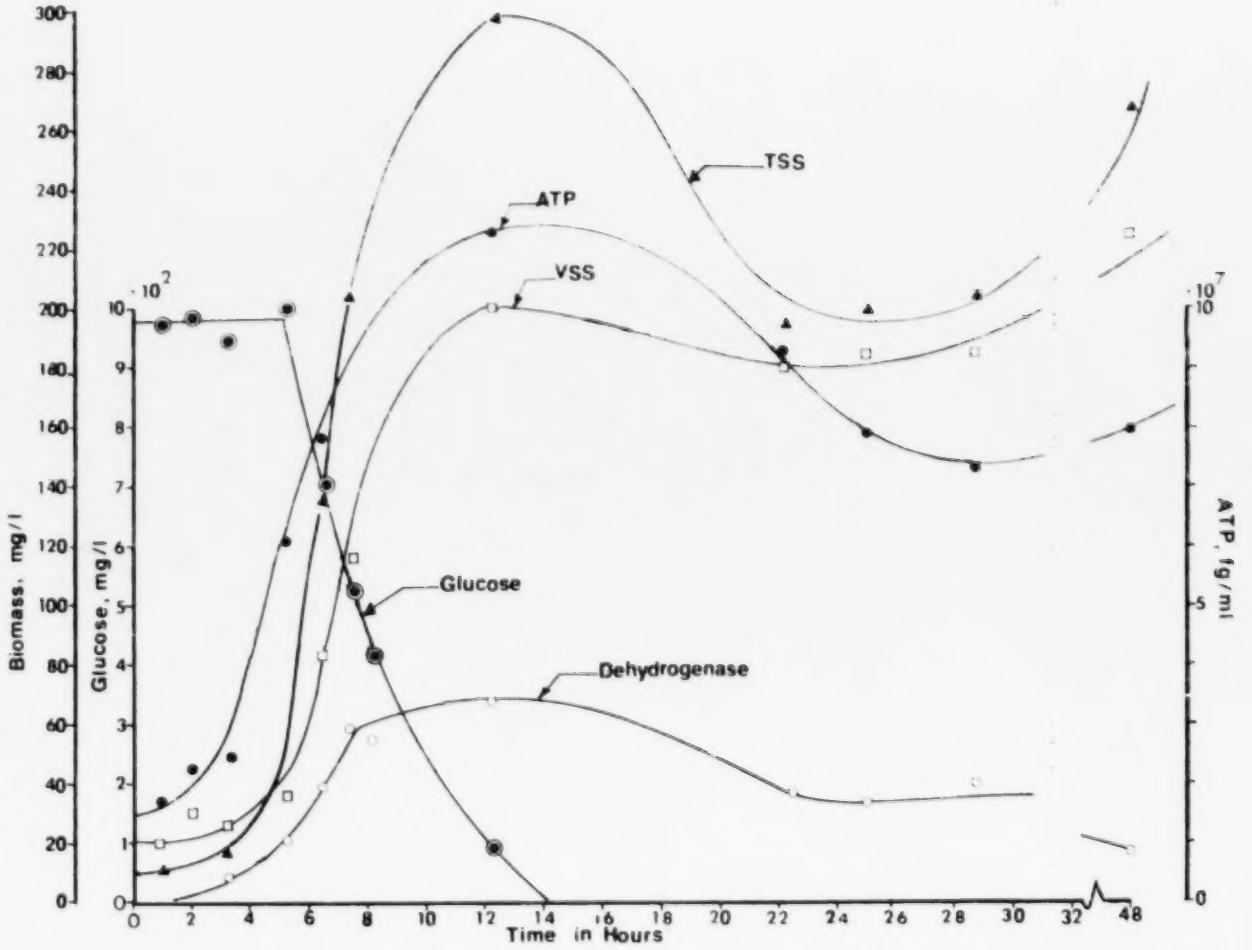

Figure 41. Nutrient Deficient Culture Batch No. 3 (C/N=30)

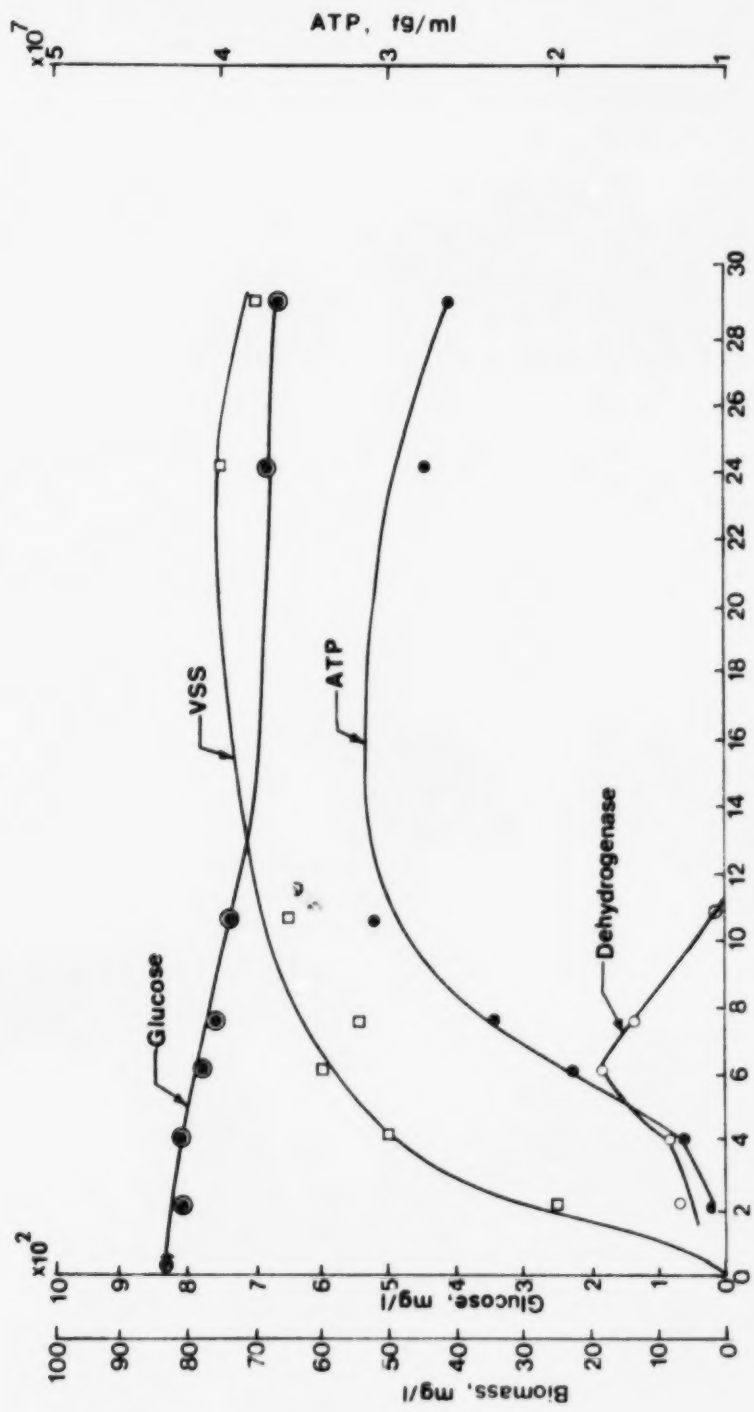

Figure 42. Nutrient Deficient Culture Batch No. 4 (C/P=150)

Cost Analysis:

The results obtained to date are encouraging with respect to the use of dehydrogenase activity as a measure of active biomass concentration in biological systems. Moreover, this analysis may prove far less tedious and costly than other methods of analysis and appears to be more reliable and interpretable than other techniques. Some comparison of the cost of analytical techniques has been included in Appendix D. It is anticipated that these studies will provide the basis for an analytical method for the determination and application of dehydrogenase activity to design and control of biological waste treatment processes.

SECTION VI

REFERENCES

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APPENDIX A

TOTAL SUSPENDED SOLIDS DETERMINATION

Procedure

1. Wash 0.45 μ white grid 47 mm Millipore filters with distilled water under vacuum for approximately two minutes in order to remove the glycerine and wetting agent and to insure a constant tare weight. Lable filters and dry filters for 30 minutes at 103°C and desiccate for 30 minutes prior to weighing.

2. Weigh and record tare weight of each filter. Store filters in desiccator prior to use.

3. Each sample is filtered through a pair of tared filters. The top filter in the pair is the test filter which will retain the solids, while the bottom filter in the pair is the control filter.

4. Using forceps, place the pair of filters on a fritted base of the filter holder so that the test filter is above the control filter. Place the funnel on top of the fritted based and clamp securely.

5. Pipet the selected volume of sample into funnel, apply vacuum to the filter, wash inside of funnel free of any attached solids, then filter to dryness.

6. Release vacuum, remove clamp, and funnel from the holder, and with forceps carefully remove and separate the pair of filters. (Use care not to disturb the surface of the test filter.) Place each filter on a teflon pad (wet filter tend to stick to glass).

7. Dry filters at 103°C for 30 minutes. Desiccate for 30 minutes after drying. Reweigh each filter and record its final weight.

8. Subtract tare weight from the final weight of each filter. The results obtained for the test filter gives the uncorrected weight of the sample solids while that for the control filter gives the gain or the loss in weight.

9. Apply control filter weight change as a correction factor to each test filter result, substracting this factor when the control filter shows a weight increase or adding the factor when the control filter shows a weight decrease.

10. Compute the dry bacterial solids concentration from the determined weight of solids and the known volume of the sample filtered.

APPENDIX B

ATP EXTRACTION PROCEDURES

1. Add 0.5 ml sample to a test tube containing 1.0 ml of 90% Dimethyl Sulfoxide (DMSO).

2. Mix 10 seconds by vortex mixer.

3. Allow to stand at room temperature for 2 minutes, the optimum recovery time.

4. Add 5.0 ml of 0.01 M morpholinopropane sulfonic acid (MOPS) buffer.

5. Mix the solution thoroughly.

6. Place tube contianing test material into ice bucket until assayed.

7. Assay the solution directly using the following formula to convert Biometer readings to units per milliliter of sample.
   Units/ml = Biometer Reading x 13

APPENDIX C

PROCEDURE FOR DEHYDROGENASE TEST

1. Set up 3 NPN tubes: one for blank and two for samples in duplicate. Pipette 8 ml of distilled water into blank tube.

2. Pipette 1 ml Tris buffer into each tube.

3. Disintegrate the sample at 30 atmospheres of nitrogen (Parr Bomb) or at 15,000 rpm in a blender for 2 minutes. Pipette 8 ml of sample into two sample tubes.

4. Incubate at 37°C and bubble nitrogen at slow rate.

5. After 10 minutes, add 1 ml TTC-glucose to all tubes and return the TTC-glucose to refrigerator.

6. Incubate for 60 minutes and continue nitrogen flow. Cover tubes with black plastic sheet.

7. After 60 minutes add 1 ml of formaldehyde. Also add 1 ml of 4 N HCI to each tube. Stop nitrogen flow.

8. Wash nitrogen purging tubes with 95% ethyl alcohol.

9. Dilute samples and blank to 50 ml mark · Mix.

10. Keep samples in darkness for 30 minutes.

11. Filter through cotton in a funnel and into cuvette (10 or 1 cm ligh path).

12. Read and note % transmittance at 483 mμ and 0.05 mm slit width.

APPENDIX D

TIME AND COST SUMMARY OF SELECTED ANALYTICAL TECHNIQUES

Analytical Technique	Time to Perform Analysis	Cost of Specialized Equipment and Reagents
Dehydrogenase Test	2 1/2 hrs. (1 hr. incubation and 30 min. color development)	Parr Bomb - $678.00 2,3,5 Triphenyltetrazolium chloride - $3.83/5g. (allows for 2500 tests)
Total Suspended Solids	1 1/2 hrs. (30 min. drying period & 30 min. in desiccator)	No special equipment.
ATP by Biometer	10 min. (30 min. for enzyme substrate and buffer preparation prior to analysis)	Reagents (substrate and buffer) (allows for 500 tests) $160 Biometer $5,000
Glucostat Test	1 1/2 hrs. (30 min incubation)	Glucostat reagents - $4.90/box of 5 sets of reagents (allows for 50 tests)
Galactostat	3 1/2 hrs. (1-1 1/2 hrs. incubation)	Galactostat reagents - $13.75/box of 5 sets of reagents (allows for 85 tests)
Coulter Counter Enumeration	3-4 hrs.	Isoton (particle-free electrolyte) $17.00/5 gal. (allows for 190 tests)
Millipore Filter Technique	20 min. (16 hr. incubation)	MF sterile filter paper - $15.00/box of 100 MF sterile plastic petri dishes $6.00/box of 100